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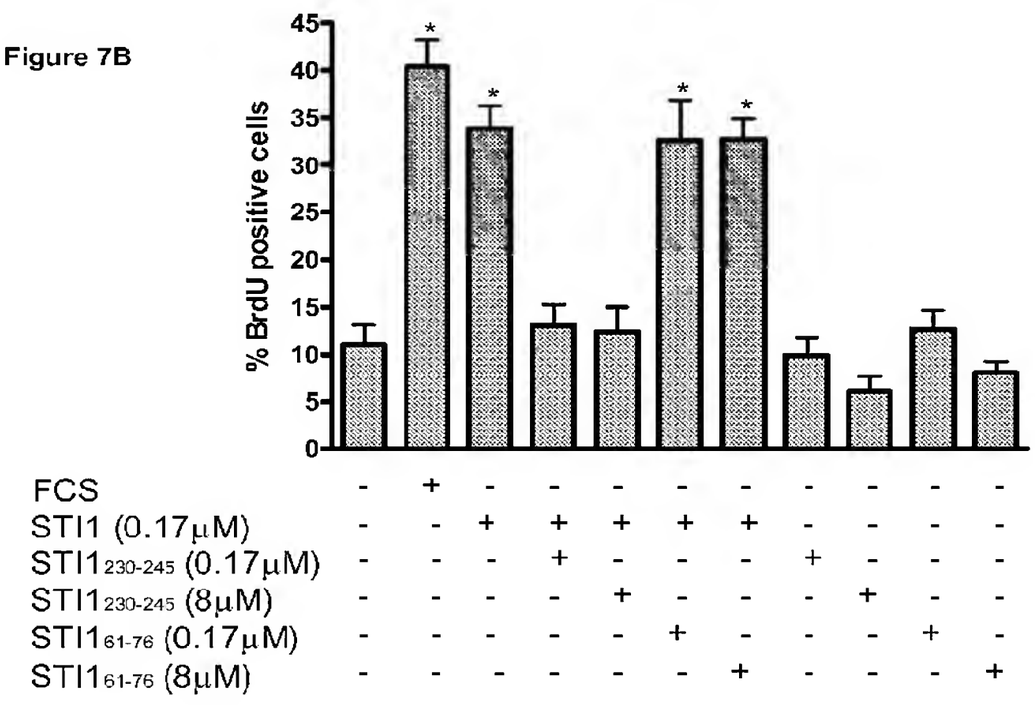
(54) **Title:** PEPTIDES AND METHODS FOR THE TREATMENT OF GLIOMAS AND OTHER CANCERS

Figure 7B



(57) **Abstract:** The invention is based on the discovery that STM /Hop promotes proliferation of human glioblastoma-derived cells but not of normal astrocytes and that the proliferation requires the binding of STM /Hop to PrP^c. The invention is directed to methods for treating cancer which rely on interfering with the Hop-PrP^c interaction and to peptides, and antibodies raised against the peptides, which directly provide that interference. The invention is further based on the discovery that $STI1_{230-245}$ peptide and its human homologue Hop23o-245 provide the desired interference with the STI1/Hop-PrP^c interaction and inhibit the STI 1/Hop-induced proliferation of glioma and glioblastoma cells. The invention is thus further directed to methods of treating cancer that employ these peptides and functional derivatives thereof, and antibodies directed to the peptides and derivatives. The invention is further directed to means of treating cancer which involve reducing the effective amount of Hop or reducing the expression of Hop. The invention is further directed to means of alleviating or eliminating the side effects of drug therapy and radiotherapy used in treating patients with brain cancers.

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PEPTIDES AND METHODS FOR THE TREATMENT OF GLIOMAS AND OTHER CANCERS

This application claims the benefit of U.S. Provisional Application No. 60/972,958, filed September 17, 2007.

BACKGROUND OF THE INVENTION

Gliomas are tumors derived from glia or their precursors within the central nervous system. Malignant gliomas, the most common subtype of primary brain tumors, are aggressive, highly invasive and neurologically destructive. Clinically, gliomas are divided into four grades and the most aggressive of these, grade IV astrocytoma or glioblastoma multiforme (GBM), is also the most common in humans (Kleihues 2000; Maher et al. 2001). Despite maximum treatment efforts, median survival of patients diagnosed with GBM ranges from 9 to 12 months, a statistic that has changed very little in decades. Primary brain tumors, like all cancers, share a relatively restricted set of characteristics crucial to their phenotype: proliferation in the absence of external growth stimuli, avoidance of apoptosis and no limits to replication, escape from both external growth-suppressive forces and the immune response, formation of new blood vessels and the ability to invade normal tissues (Hanahan and Weinberg 2000). Furthermore, despite their striking heterogeneity, common alterations in specific cellular signal transduction pathways occur within most GBMs. Deregulation of signal transduction, which accounts for aberrant responses to distinct soluble factors, is also a common feature of these tumors, and modulation of signaling pathways has become an option for targeted therapies (Sebolt-Leopold and Herrera 2004).

Previous work from our group (Zanata et al. 2002) identified the co-chaperone stress-inducible phosphoprotein 1 (STI1) as a cell-surface ligand for the membrane glycosylphosphatidylinositol (GPI) anchored cellular prion (PrP^C), which leads to the activation of several signal transduction pathways, some of which modulate cell survival. Stress-inducible phosphoprotein 1 (STI1), also referred to, in the case of the human homologue, as Hop (Hsp70/Hsp90

organizing protein), is a 66kDa protein first identified in yeast and originally described as a co-chaperone that binds to both Hsp70 and Hsp90, and regulates their activities (Chen and Smith 1998; Nicolet and Craig 1989; Song and Masison 2005). Due to the 98% sequence homology between the mouse (STI1) and human (Hop) molecules (Table 1), the term STI1/Hop will be used throughout this disclosure as the designation for the protein. In cases where the intention is to specify either the mouse or human homologue, the respective designation STI1 or Hop alone will be made.

STI1/Hop is present in diverse cellular locations, exists within nuclear transcription complexes, is able to move dynamically between the cytoplasm and the nucleus (Odunuga et al. 2004) and although it lacks a transmembrane domain or a signal peptide for membrane transport, it is also present at the cell surface (Martins et al. 1997; Zanata et al. 2002). In fact, many proteins expected to be confined in the cytoplasm are also at the cell surface where they play specific functions, in particular as receptors for plasma proteins (Nickel 2005). Previous work had already showed that STI1/Hop involvement in Hsp90-independent complexes relates to diverse cellular events such as transcription, protein folding and translocation, viral replication, signal transduction and cell division (Odunuga et al. 2004). STI1/Hop was shown to be secreted by normal astrocytes (Lima et al. 2007) and by HT-1080 fibrosarcoma cells together with other chaperones and co-chaperones, suggesting that these proteins may form extracellular active Hsp90 complexes related to MMP2 (metalloproteinase 2) activation and consequent tumor invasiveness (Eustace and Jay 2004; Eustace et al. 2004). Another study also related the activity of Hsp90, an STI1/Hop partner, to a molecular mechanism of tumor response selectivity to geldanamycin (Kamal et al. 2003).

Previous work from our group showed that a cellular prion-binding peptide designed on the basis of the complementary hydropathy theory (Boquet et al. 1995; Brentani 1988; Martins et al. 1997), later identified as a domain of STI1/Hop (Zanata et al. 2002), was able to activate the PKA and Erk signaling pathways, with the former being associated with cell survival in retinal explants (Chiarini et al. 2002). In addition, recombinant STI1/Hop was reported to modulate retinal proliferation and cell death (Arruda-Carvalho et al. 2007), to trigger neuroprotection and neuritogenesis in hippocampal neurons through

PKA and MAPK pathways, respectively (Lopes et al. 2005) and to induce endocytosis-dependent MAPK signaling (Americo et al. 2007; Caetano et al. 2008).

BRIEF SUMMARY OF THE INVENTION

The invention is based on the discovery that STI1 promotes proliferation of human glioblastoma-derived cells but not of normal astrocytes and that the proliferation requires the binding of STI1/Hop to PrP^C. The invention is directed to methods for treating cancer which rely on interfering with the STI1/Hop-PrP^C interaction and to peptides, and antibodies raised against the peptides, which directly provide that interference.

The invention is further based on the discovery that $STI1_{230-245}$ peptide and its human homologue $Hop_{230-245}$ provide the desired interference with the STI1/Hop-PrP^C interaction and inhibit the STI1/Hop-induced proliferation of glioma and glioblastoma cells. The invention is thus further directed to methods of treating cancer that employ these peptides and functional derivatives thereof, and antibodies directed to the peptides and derivatives.

The invention is further directed to means of treating cancer which involve reducing the effective amount of STI1/Hop or reducing the expression of STI1/Hop.

The invention is still further directed to use of the disclosed peptides in diminishing and even eliminating the side effects of standard treatments given patients with brain cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1D depict the results of experiments showing secretion of Hop by A172 cells and the consequent proliferation of cells.

Figures 2A-I depict the results of experiments showing the involvement of the MAPK and PI3K pathways in STI1-induced glioma-cell proliferation.

Figure 3 is a graphic depiction of STI1-induced proliferation in distinct glioma cell lines.

Figures 4A-D depict the results of experiments showing that STI1 does not induce proliferation of normal glia.

Figures 5A and B are graphic depictions of experimental results showing that the PrP^C binding site of STI1 is necessary for STI1-induced proliferation.

Figure 6 is a graphic depiction of experimental results showing that $STI1_{230-245}$ peptide does not promote proliferation of A172 cells.

Figures 7A and B are graphic depictions of experimental results showing that $STI1_{230-245}$ peptide abrogates STI1-induced proliferation of glioma cell lines.

Figure 8 is a graphic depiction of experimental results showing that $Hop_{230-245}$ peptide inhibits STI1-induced proliferation of A172 cells.

Figure 9 is a graphic depiction of the results of experiments designed to test the therapeutic efficacy of TAT-conjugated forms of $STI1_{230-245}$ and $Hop_{230-245}$ peptides in inhibiting STI1-induced proliferation.

Figure 10 shows by fluorescence microscope imaging, tests of the ability of dansylated TAT-$STI1_{230-245}$ and TAT-$STI1_{422-437}$ peptides to cross the cell membrane.

Figure 11 is a graphic depiction of the results of experiments designed to test the effect of $STI1_{230-245}$, $Hop_{230-245}$ and their TAT-associated forms on memory formation and to test the efficacy of the peptides as neuroprotective agents.

Figure 12 is a graphic depiction of the results of monitoring Hop gene expression in various glioblastoma samples and normal brain tissue.

DETAILED DESCRIPTION OF THE INVENTION

MATERIALS AND METHODS

Chemicals

U0126, LY294002 and Forskolin were obtained from Sigma (USA). Some of the drug preparations were made in cell-culture-grade dimethylsulfoxide (DMSO) (Sigma, USA). The final concentrations of DMSO in the culture medium in all experiments were a maximum of 0.4% (v/v). [^{3}H]-thymidine was obtained from IPEN/CNEN (Brazil). Rabbit IgG was obtained from Sigma (USA). Polyclonal antibodies recognizing total Akt, phosphorylated Akt (Ser 473), phosphorylated p44/p42 MAPK, tubulin and peroxidase-conjugated anti-rabbit IgG secondary antibody were obtained from Cell Signaling (USA). Polyclonal antibody recognizing Erk2 was obtained from Santa Cruz (USA). Polyclonal antibody recognizing STI1/Hop was obtained from Bethyl (USA) (Zanata et al. 2002). Polyclonal antibody recognizing GFAP was obtained from Dako (USA). A polyclonal antibody recognizing PrP^C raised in *Prnp* knock-out mice was produced at Ludwig Institute for Cancer Research, São Paulo (Brazil) (Lee et al. 2001). Chemicals and reagents were analytical grade or better.

Maintenance of cell lines

The A172, U87-MG, C6 and MCF7 tumor-cell lines were obtained from ATCC (American Type Culture Collection) and were grown and maintained in Dulbecco's modified Eagle medium-F12, supplemented with glucose (33mM, Merck), glutamine (2mM, Calbiochem), sodium bicarbonate (3mM, Merck), penicillin/streptomycin (0.5 mg/ ml), Fungizone (2.5 ug/ml, Squibb) and fetal bovine serum 10% (v/v) (Gibco). Culture flasks were maintained at 37° C in 5% CO_2 and 95% air. Exponentially growing cells were detached from the culture flasks with 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) and seeded at different densities depending on the assay.

Astrocyte cultures

Primary astrocytes were obtained from Wistar rats. The cerebral hemispheres were dissected and the meninges carefully removed. During dissection the tissue was maintained in a PBS-0.6% glucose solution. Thereafter the tissue was mechanically dissociated and centrifuged in a clinical centrifuge. The supernatant was removed, the pellet resuspended in DMEM-F12 with 10% FBS and the cells seeded in flask cultures. The culture media was changed every other day. Once the cultures became semi-confluent, they were washed with PBS, detached from the culture flasks with 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) and seeded at different densities depending on the assay.

Western blotting

After the treatments described herein, cells were washed twice with cold phosphate-buffered saline (PBS) and lysed in 1% NP40, 1% Triton X-100, 1% Sodium Deoxycholate, 10mM Tris-HCl pH 7.5, 100mM NaCl, 0.1% SDS, 5mM EDTA, supplemented with Complete protease and phosphatase inhibitory cocktails (Roche, USA). Samples (30-40 µg) were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were blocked with 5% nonfat milk in Tris-buffered saline, 0.1% Tween-20 (TBS-T) for one hour, incubated with primary antibodies (STI1/Hop 0.5 µg/ml purified IgG, phospho-Erk 1:1000, phospho-Akt 1:1000, Erk2 1:5000, Akt 1:1000, tubulin 1:1000) overnight at 4° C, washed with TBS-T and incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000) for one hour. The reactions were developed using enhanced chemiluminescence (Pierce, USA). The conditioned media (CM) from A172 cells were filtered to remove cell debris and concentrated in an Amicon apparatus (Amicon, USA) before electrophoresis. Western blotting assays for CM were conducted using anti-STI1/Hop (0.5µg/ml) antibody.

Immunodepletion assay

A172-CM was incubated with rabbit anti-STI1/Hop antibody (4µg/ml) overnight at 4°C, mixed with CL-4B Sepharose (Pharmacia, USA) for 2h at 4°C

and then centrifuged. The pellet and the supernatant (CM depleted of STI1/Hop) were analyzed for the presence of STI1/Hop.

Immunocytochemistry

A172 tumor cells and rat astrocytes were seeded at 5 x 10^4 cells per well on glass slides in 24-well plates and cultured for 24 hours in serum-free culture media. Then the culture media was removed and the cells were fixed with 4% paraformaldehyde in phosphate buffer for 30 seconds. Fixed cells were washed with PBS and then incubated with a solution of 1% bovine serum albumin (BSA) in PBS for 1 hour. Thereafter, the cells were incubated with serum anti-STI1/Hop (1:200 in 1%BSA) or anti-GFAP (1:1000 in 1% BSA) antibodies for 16 hours at 4° C, washed again with PBS and incubated with the Cy-3- or FITC-conjugated secondary antibodies (1:500 in PBS) for 3 hr. Then the cells were washed with PBS, stained with DAPI (Sigma), washed again and mounted. For negative controls, cells received similar treatment but the primary antibodies, anti-STI1/Hop or anti-GFAP, were omitted.

Flow cytometry assay

Cells were grown and detached as described above. Then at least 10^6 cells were collected, centrifuged, resuspended in a PBS/BSA 0.5% solution and incubated with a polyclonal antibody anti-PrP^C (Lee et al. 2001) (1:100) for 1 hr at room temperature (RT). Thereafter cells were washed three times with PBS, incubated with a secondary antibody for 1 hr at RT, and assayed in a BD FACScan using the Lysis II program.

Conditioned media

Semi-confluent A172 cell cultures maintained in 75cm^2 culture flasks were washed once with PBS and three times with serum-free media. Cells were cultured for 48 hr in serum-free culture media. After this period the conditioned media was collected and subjected to 5 min. centrifugation at 1500 rpm in a standard clinical centrifuge. The supernatant was collected and stored at -70°C.

Cell viability

Cells were cultured for 24 hr in 24-well plates in serum-free media and subjected to identical treatments as in [^{3}H]-thymidine incorporation assays. Thereafter, the culture media was removed; the cells were incubated with Trypan Blue 0.2% (v/v) in PBS for 1 min. and then washed with PBS. A minimum of 5 x 10^2 cells per well in randomly selected fields were counted.

Expression and purification of STI1

Recombinant wild-type and mutant STI1 were obtained as previously described (Lopes et al. 2005; Zanata et al. 2002). Briefly, protein expression was induced by 1.5mM isopropyl-ß-D-thiogalactopyranoside (IPTG) for 4hr in *Escherichia Coli* DH-5α cells (Stratagene) containing the expression vector His_6-STI1. Cells were resuspended in lysis buffer (Na_2HPO_4 50mM, NaCl 300mM, pH 8.0) containing protease inhibitors, and subjected to freeze-thawing cycles. Protein was purified using Ni-NTA-agarose (Qiagen) in accord with manufacturer's instructions.

Treatments

For [^{3}H]-thymidine incorporation and cell viability assays, U0126 (10 μM), LY294002 (5 μM), Forskolin (10 μM) (Chen et al. 2003, Lee et al 2005, Shingu et al. 2003), recombinant STI1 (170 nM) and DMSO (0.4%) were added at the beginning of the 24-hr period. For western blots, cells were subjected to STI1/Hop, U0126 and LY294002 treatments as indicated in the figure legends. For co-treatments, the inhibitors were added 10 minutes prior to STI1.

Densitometries

Densitometric analyses of the immunoblots were performed using the ImageQuant software. The ratio between phospho-specific bands and their respective loading controls was calculated and the results were normalized to the respective control groups.

Proliferation Assays

Proliferation assays were conducted using [^{3}H]-thymidine incorporation or BrdU incorporation.

a. [^{3}H]-thymidine incorporation assays

Tumor cells were subjected to a "starvation period," i.e., they were seeded at 10^4 cells per well in DMEM-F12 serum-free in 48-well plates. Rat astrocytes were seeded at 4 x 10^4 cells per well in DMEM-F12 serum-free or 5% FBS in 48-well plates. After 3 hours, the different compounds with which the cells were to be treated were added. After 18 hours of treatment, a [^{3}H]-thymidine 6-hour pulse was added. At the end of this 24-hour period, the medium was carefully removed and 300 μl of ice-cold 10% trichloroacetic acid was added. Cells were harvested and [^{3}H]-thymidine incorporation was measured with a scintillation counter.

b. BrdU incorporation assays

b.1 *A172 cell line*

Cells were plated overnight on 24-well plates with glass coverslips, at a density of 1x10^4 cells per well, in DMEM high glucose plus 10% FCS at 37°C. Plates were washed 3 times with PBS and were maintained in DMEM without FCS for 30 hours. Cells were then treated for 18 hours with DMEM/10% FCS or DMEM plus mouse recombinant STI1 (170nM); STI1 (170nM) plus $STI1_{230-245}$ peptide (170nM); or STI1 (170nM) plus $STI1_{61-76}$ irrelevant peptide (170nM). Thirty minutes before the end of treatment, cells received a pulse of BrdU (35μM) and were fixed with 4% paraformaldehyde.

b.2 *U87MG cell line*

Cells were plated overnight on 24-well plates with glass cover slips, at a density of 1.5x10^4 cells per well, with DMEM with 10% FCS at 37°C. Plates were washed 3 times with PBS and were maintained in DMEM F12 for 48 hours. Cells were then treated for 24 hours with DMEM/10% FCS or DMEM plus mouse recombinant STI1 (170nM); recombinant STI1 (170nM) plus mouse

peptide $STI1_{230-245}$ (ELGNDAYKKKDFD**K**AL) or its human homologue peptide $Hop_{230-245}$ (ELGNDAYKKKDFD**T**AL) (170nM or 8μM); STI1 (170nM) plus $STI1/Hop_{61-76}$ (GCKTVDLKPDWGKGYS) irrelevant peptide (170nM or 8μM); or each one of the peptides alone. Two hours before the end of treatment, cells received a pulse of BrdU (35μM) pulse and were fixed with 4% paraformaldehyde.

b.3 Immunofluorescence assay for BrdU incorporation

Coverslips were treated with 2N HCl for 30 minutes. The reagent was then removed and borate buffer applied (boric acid 0.1M, sodium hydroxide 0.15M), pH 8.4, for 10 minutes. Then, cells were treated with PBS plus 0.2% Triton X-100 for 15 minutes. The blocking step was made with PBS/0.2% Triton with 20% horse serum, for 1 hour. Primary anti-BrdU biotinylated antibodies (1:100) were applied overnight (diluted in PBS/0.2% Triton with 1% horse serum). Coverslips were washed 3 times with PBS and incubated for one hour with Strepta-Alexa 488 and DAPI reagents (1:1000), washed more 3 times with PBS and assembled to slides.

Cell counting

There were taken at least four microscopic fields of each treatment on WB filter, ranging from 330-385nm (DAPI), plus their respective BrdU images, on WIB filter, 450-480nm using DP controller software, Olympus. Picture files were analyzed on ImageJ software, and the percentage of BrdU positive nuclei in respect to total nuclei number (DAPI) was calculated using the Analyze Particles tool.

Tissue samples

Fresh surgical samples of glioblastoma and of non-tumor tissue of the CNS (temporal lobectomy from epilepsy surgeries) were macrodissected and immediately snap-frozen in liquid nitrogen upon surgical removal. Necrotic and non-neoplastic areas were removed from the frozen block and the tumor tissue was microdissected prior to the RNA extraction procedure.

For quantitative real time RT-PCR, 17 samples of non-tumor and 76 glioblastomas from humans were evaluated.

Total RNA isolation and cDNA synthesis

Total RNA was extracted from normal and tumor tissues using guanidine isothiocyanate. Conventional reverse transcription was performed to obtain single-strand cDNA for real time RT-PCR.

Quantitative real-time RT-PCR

The STI1/Hop expression levels were determined by real time PCR analysis. Primers were designed to amplify a DNA fragment of 101-bp length. Primer sequences were as follows (5' to 3'): F:CCTGGGCACGAAACTACAAGA, R: GCAATCTCTTCCTCCTCATCC. All primers were synthesized by Sigma.

The minimum concentration of primers was determined by the lowest threshold cycle and maximum amplification efficiency while minimizing nonspecific amplification. Analysis of DNA melting curves demonstrated a single peak for both primers. The reactions consisted of: 3µl of primer mixture (final concentration of 100nM), 3µl of cDNA sample, and 6µl SYBR Green I Master Mix (Applied Biosystems). Reactions were run on an ABI Prism 5700 sequence detector (Applied Biosystems). DNA melting curve analysis showed a single peak for the STI1/Hop amplified product. Quantitative data was normalized relative to the internal housekeeping control (BCRP- Breast cancer resistance protein; HPRT-hypoxanthine-guanine phosphoribosyltransferase and GUSβ – β glucuronidase).

The $1{,}73^{-\Delta\Delta Ct}$ equation was applied to calculate the relative expression of Hop in tumor samples versus the median of normal CNS tissues, where ΔCt = Ct gene - Ct normalized, and $\Delta\Delta Ct = \Delta Ct$ tumor - mean ΔCt normal tissues.

Behavioral training of animals in long-term memory tests

Rats were trained in a one-trial, step-down IA paradigm, a hippocampal-dependent learning task in which stepping down from a platform present in a given context is associated with a foot shock, resulting in an increase in step-

down latency when the animal is brought back to the training chamber for a retention test (Cammarota et al., 2004). The IA apparatus was a 50 X 25 X 25 cm Plexiglas box with a 5-cm-high, 8-cm-wide and 25-cm-long platform on the left end of a series of bronze bars that constitutes the floor of the box. During training, each animal was gently placed on the platform facing the left rear corner of the training box. When an animal stepped down and placed all four of its paws on the grid, it received a 2-s, 0.5-mA foot shock and was then immediately withdrawn from the training box. Animals were replaced in the IA box for retention testing 90 min (for short-term memory (STM)), or 24 h later (for long-term memory (LTM)); their latency to step down onto the grid was recorded. The difference between the training and test-session step-down latencies was taken as a measure of retention. Better memory for the training was inferred from longer retention latencies (Bernabeu et al., 1997; Izquierdo et al., 1997). The maximum retention latency allowed was 180 s, at which time the animal was taken out of the IA box if it had not stepped down and given a score of 180 s. Full details of the experimental protocols can be found in Coitinho et al., 2007.

Statistical analysis

The number of experimental replicates for each of the studies shown in Figures 1-6 is given in the respective figure legends. Data were analyzed by Student's t-test when two groups are compared or ANOVA followed by *post-hoc* comparisons (Tukey's test) when multiple groups are compared.

In proliferation assays, the mean values of at least three independent datasets are shown in the figures; the error bars represent standard error of measurement (SEM). ANOVA followed by Tukey-HSD or Dunnets tests were used for multiple comparisons. Results were considered statistically significant when p was < 0.05.

For quantitative RT-PCR statistical analysis was applied to gene expression data obtained from both glioblastomas and normal CNS tissues. The Mann-Whitney test was used and the results were considered statistically significant when p was < 0.05.

The data obtained in tests of the effect of peptides on rat long-term memory are presented as medians ± interquartile range and were analyzed by

the Kruskal-Wallis nonparametric test followed by Dunn's post-hoc (n=10 to 13 animals for each treatment group). In all comparisons, $p < 0.05$ was taken as a significant difference.

RESULTS

Hop is secreted by A172 human glioblastoma cell line and induces proliferation.

We first tested for the expression of Hop in the A172 human GBM cell line. Western blot assays of A172 total lysates probed for Hop showed a single band at the expected molecular weight (66kDa) (Fig. 1*A*), and immunocytochemistry of cells cultured in serum-free media and fixed with paraformaldehyde showed extensive Hop immunolabeling (Fig. 1*B*).

The cellular secretion of Hsp-90 as well as STI1/Hop has been previously described (Eustace and Jay 2004; Eustace et al. 2004) and results from our group (Lima et al. 2007) demonstrated that STI1/Hop is secreted from primary astrocyte cultures. We conducted western blots and immunoprecipitation assays and demonstrated the presence of Hop in conditioned medium from A172 cells (Fig. 1*C*; CM). Hop was immunoprecipitated from CM using specific antibodies (Fig. 1*C* pellet) and depleted from the immunoprecipitation supernatant material (Fig. 1*C*; depleted). The lack of CD44 in the conditioned medium also confirmed the absence of cell lysis. Therefore, these data demonstrated that Hop is secreted from the A172 glioma cell line.

To test whether STI1/Hop modulates proliferation of a GBM cell line, we assayed [^{3}H]-thymidine incorporation in A172 cells cultured in serum-free media. Proliferation was determined by quantitative measurement of [3H]-thymidine incorporation (6.7μCi/ml, 6-hour pulse) and the results were normalized with respect to the rate of proliferation (100%) in serum-free media (CTR). A172 cells treated with recombinant STI1 (170 nM) showed a marked increase in the uptake of thymidine, as compared with control (Fig. 1*D*), indicating that STI1/Hop induces proliferation of this cell line. Dose- response curves demonstrated that the STI1/Hop effect was maximum at 170 nM (Fig. 5A).

MAPK and PI3K signaling pathways are involved in the STI1-induced proliferation of glioma cells.

MAPK and PI3K signaling pathways commonly relate to cell proliferation and are often deregulated in cancer. In order to investigate the involvement of these pathways in STI1-induced proliferation, A172 cells were treated with STI1 in the presence of either U0126, an inhibitor of the Erk-activating kinase MEK, or LY294002, an inhibitor of PI3K. These drugs completely abolished STI1-induced proliferation, which suggests the involvement of both pathways (Fig. 2*A*) in this phenotype. Cell viability was always higher than 95%, ruling out possible cytotoxic effects of the inhibitors as the cause of the proliferation blockade (Fig. 2*B*).

Western blots of lysates from A172 cells cultured in serum-free media confirmed that treatment with STI1 induced activation of Erk and Akt and that this effect was abolished by co-treatment with U0126 or LY294002, respectively (Figs. 2*C, D* and 2*E, F*). Interestingly, STI1 co-treatment with LY294002 led to an increase in the phosphorylation of Erk (Figs. 2*C, D*; lane 4) as compared with cells treated only with STI1 (Fig. 2*C, D*; lane 2).

Paradoxical effects of MAPK activation regarding not only its intensity, but also its duration have already been described (Marshall 1995; Sebolt-Leopold and Herrera 2004; Sewing et al. 1997; Sharrocks 2006). In this context, we assayed for Erk activation induced by treatment with STI1 alone, STI1 in the presence of LY294002 and LY294002 alone for 5, 35 and 60 minutes (Figs. 2*G, H*). Although the levels of Erk activation in cells treated for 5 min. with LY294002 (lane 8) were similar to those in cells treated with STI1 (lane 2), Erk activity induced by the PI3K inhibitor was much more durable, lasting for at least one hour (lanes 8-10), as opposed to that seen in STI1-treated cells (lanes 2-4). STI1 treatment in the presence of the PI3K inhibitor led to an increase in the intensity and duration of Erk activity (lanes 5-7) as compared to that seen in cells treated with STI1 alone (lanes 2- 4).

STI1/Hop-induced neuroprotection was previously related to PKA pathway activation (Chiarini et al. 2002; Lopes et al. 2005). In this context we tested for the effect of forskolin, an activator of adenylyl cyclase, upon

incorporation of thymidine by A172 cells (Fig. 2*I*). Even when cultured in serum-free media, cells treated with forskolin showed a marked decrease in DNA synthesis, as compared to control group. This result suggests that the involvement of PKA in STI1/Hop-induced proliferation is unlikely.

STI1/Hop modulates the proliferation of distinct glioma cell lines.

Although gliomas are classified in different groups based on histological features, it is known that these tumors are constituted by a heterogeneous set of cell populations, which renders each tumor a unique pathologic process and prevents the development of broadly effective therapeutic regimens. To address the generality of the effect of STI1/Hop, we treated distinct tumor cell lines with STI1 and assayed for thymidine incorporation (Fig. 3). Similarly to A172 cells, both C6 and U87-MG cell lines, respectively, a rat glioma and a human GBM, were responsive to STI1 treatment, although with different intensities. On the other hand, the MCF-7 cell line, a breast adenocarcinoma, was insensitive to STI1 treatment.

STI1/Hop does not induce proliferation of normal astrocytes.

To compare with the response of the GBM cell line, normal astrocytes obtained from neonate rats cultured in serum-free media (Fig. 4*A*) or in media supplemented with FBS 5% (Fig. 4*B*) were treated with STI1, and assayed for thymidine incorporation. STI1 had no effect upon the low level of proliferation of the astrocytes cultured in serum-free medium. However, STI1 treatment produced a small but statistically significant decrease in the proliferation of astrocytes cultured in FBS 5%. The effect of FBS 5% upon astrocyte proliferation is shown in Fig. 4*C*. Immunocytochemistry assays confirmed the expression of GFAP in these astrocytes (Fig. 4*D*).

The PrP^C binding site of STI1/Hop is necessary for STI1-induced proliferation of A172 cells

Cellular prion (PrP^C) was previously described as an STI1/Hop receptor and also related to STI1/Hop-induced neuroprotection and differentiation (Chiarini et al. 2002; Lopes et al. 2005; Zanata et al. 2002). To investigate a possible role of PrP^C in the proliferative effect of STI1/Hop, we performed

thymidine incorporation assays in A172 cells treated with distinct concentrations of a mouse STI1 mutant lacking residues 230-245 (STI1$\Delta_{230-245}$) (Lopes et al. 2005) whose deleted domain was previously characterized as the PrP^C binding site (Zanata et al. 2002). While wild-type STI1 promoted proliferation of glioma cells, the mutant STI1 did not promote proliferation (Fig. 5*A*). Flow cytometry confirmed the presence of PrP^C at the surface of viable A172 cells (Fig. 5*B*). These data indicate a role for PrP^C in STI1/Hop-induced proliferation of A172 cells.

The use of $STI1_{230-245}$ peptide to block STI1/Hop-dependent glioma proliferation

In light of the observations disclosed above, we have identified mouse $STI1_{230-245}$ and other related peptides as candidates for effectively inhibiting the proliferation of human gliomas and other cancers triggered by the interaction between STI1/Hop and PrP^C. Accordingly, we undertook various additional investigations into the effect of such peptides on glioma-cell proliferation.

The proliferation was assessed by bromodeoxyuridine (BrdU) incorporation, followed by immunofluorescence imaging and cell counting. The "starvation" period confers a better interval for observation of the effects of the studied factor (STI1/Hop) and the hypothetical inhibitors $STI1_{230-245}$ and $Hop_{230-245}$ peptides. Additionally, the BrdU assay affords a more reliable proliferation evaluation, in which it is possible to observe the morphology and integrity of cells, and documentation (microscope imaging). While a thymidine incorporation assay best exhibits speed of DNA synthesis, by indirect CPM counting, a BrdU incorporation assay is more objective, allowing the measurement of the number of cells synthesizing DNA and deduction of the percentage of cells under proliferation. For these reasons, we selected the BrdU/DAPI ratio of positive nuclei as the analysis method.

$STI1_{230-245}$ peptide abrogates STI1-induced proliferation

Similarly to the previously described findings (Erlich et al., 2007), STI1 was observed to produce a 1.5-fold increase on A172 cell proliferation. On the other hand, mouse $STI1_{230-245}$ peptide (ELGNDAYKKKDFD**K**AL) at the same

concentration of STI1 (170nM) inhibited STI1-induced proliferation, while N-terminus irrelevant STI1/Hop peptide ($STI1/Hop_{61-76}$) had no effect on STI1-mediated proliferation (Fig. 7A).

Figure 6 depicts the results of what served as a control study; the effect of $STI1_{230-245}$ alone on proliferation of A172 cells was monitored. As can be seen from this figure, $STI1_{230-245}$ was unable by itself to promote proliferation of A172 cells. It can also be seen in Figure 6 that N-terminal irrelevant peptide $STI1_{61-76}$ did not enhance proliferation.

We observed that STI1 treatment promoted a 3-fold increase of U87MG proliferation, which was blocked by the $STI1_{230-245}$ peptide at 170nM and 8μM but not by the irrelevant $STI1/Hop_{61-76}$ peptide. The sequences of the $STI1_{61-76}$ and Hop_{61-76} peptides are the same (see Table 1). Experimental data are summarized in Figure 7B.

U87MG cells presented a slower doubling time (48 hours) than A172 and were very sensitive to serum starvation. Thus they were starved in DMEM F12 for this purpose (Brockmann et al., 2003).

$Hop_{230-245}$ peptide inhibits STI1-induced proliferation

Furthermore, we addressed whether the human homologue of mouse $STI1_{230-245}$ peptide — $Hop_{230-245}$ (ELGNDAYKKKDFD**T**AL) — could modulate proliferation in U87MG cells. The homology between STI1 and Hop peptides is shown in the side-by-side comparison of the two full sequences in Table 1.

The data in Figure 8 show that $Hop_{230-245}$ peptide, as well as its mouse counterpart ($STI1_{230-245}$), were able to abrogate the cell proliferation mediated by STI1. The N-terminal irrelevant peptide, $STI1/Hop_{61-76}$, had no effect upon proliferation mediated by STI1.

Taken together, these data validate the use of the human counterpart of $STI1_{230-245}$ peptide ($Hop_{230-245}$) to block STI1-induced proliferation.

Table 1

Homology between STI1 and Hop amino acid sequences (98%)

HUMAN - Hop
MOUSE – STI1

1 MEQVNELKEK GNKALSVGNI DDALQCYSEA IKLDPHNHVL
YSNRSAAYAK KGDYQKAYED
1 MEQVNELKEK GNKALSAGNI DDALQCYSEA IKLDPQNHVL
YSNRSAAYAK KGDYQKAYED
61 GCKTVDLKPD WGKGYSRKAA ALEFLNRFEE AKRTYEEGLK
HEANNPQLKE GLQNMEARLA
61 GCKTVDLKPD WGKGYSRKAA ALEFLNRFEE AKRTYEEGLK
HEANNLQLKE GLQNMEARLA
121 ERKFMNPFNM PNLYQKLESD PRTRTLLSDP TYRELIEQLR
NKPSDLGTKL QDPRIMTTLS
121 ERKFMNPFNL PNLYQKLEND PRTRSLLSDP TYRELIEQLQ
NKPSDLGTKL QDPRVMTTLS
181 VLLGVDLGSM DEEEEIATPP PPPPPKKETK PEPMEEDLPE
NKKQALKEKE LGNDAYKKKD
181 VLLGVDLGSM DEEEEAATPP PPPPPKKEPK PEPMEEDLPE
NKKQALKEKE LGNDAYKKKD
241 FDTALKHYDK AKELDPTNMT YITNQAAVYF EKGDYNKCRE
LCEKAIEVGR ENREDYRQIA
241 FDKALKHYDR AKELDPTNMT YITNQAAVHF EKGDYNKCRE
LCEKAIEVGR ENREDYRQIA
301 KAYARIGNSY FKEEKYKDAI HFYNKSLAEH RTPDVLKKCQ
QAEKILKEQE RLAYINPDLA
301 KAYARIGNSY FKEEKYKDAI HFYNKSLAEH RTPDVLKKCQ
QAEKILKEQE RLAYINPDLA
361 LEEKNKGNEC FQKGDYPQAM KHYTEAIKRN PKDAKLYSNR
AACYTKLLEF QLALKDCEEC
361 LEEKNKGNEC FQKGDYPQAM KHYTEAIKRN PRDAKLYSNR
AACYTKLLEF QLALKDCEEC
421 IQLEPTFIKG YTRKAAALEA MKDYTKAMDV YQKALDLDSS
CKEAADGYQR CMMAQYNRHD
421 IQLEPTFIKG YTRKAAALEA MKDYTKAMDV YQKALDLDSS
CKEAADGYQR CMMAQYNRHD
481 SPEDVKRRAM ADPEVQQIMS DPAMRLILEQ MQKDPQALSE
HLKNPVIAQK IQKLMDVGLI
481 SPEDVKRRAM ADPEVQQIMS DPAMRLILEQ MQKDPQALSE
HLKNPVIAQK IQKLMDVGLI
541 AIR
541 AIR

Therapeutic use of STI1/Hop$_{230-245}$ peptide to treat GBM.

TAT-STI1$_{230-245}$ and TAT-Hop$_{230-245}$ peptides are able to block cell proliferation mediated by STI1

Since a primary objective was to implicate STI1/Hop230-245 peptides as candidates for a therapeutic approach to GBM, an alternative method for attaining optimal distribution on the brain and for reaching tumor cells became crucial. Therefore, for this purpose, STI1 and Hop peptides were conjugated to a TAT peptide sequence (YGRKKRRQRRR), a membrane transduction domain of the HIV-1 Tat protein which permits proteins or peptides to cross the blood-brain barrier (Fawell et al., 2006; Cai et al., 2006). The biological activity of the TAT-STI1$_{230-245}$ (YGRKKRRQRRRELGNDAYKKKDFDKAL) and TAT-Hop$_{230-245}$ (YGRKKRRQRRRELGNDAYKKKDFDTAL) peptides on U87 proliferation mediated by STI1/Hop was tested. Figure 9 shows that similarly to the STI1$_{230-245}$ and Hop$_{230-245}$ peptides, TAT-STI1$_{230-245}$ and TAT-Hop$_{230-245}$ peptides were able to block cell proliferation mediated by STI1. Indeed, it was observed that the fusion of TAT peptide to the STI1$_{230-245}$ and Hop$_{230-245}$ peptides did not change their inhibitory activity on STI1-mediated glioblastoma-cell proliferation.

The TAT peptide allows the STI1$_{230-245}$ peptide to cross the cell membrane

Another requirement for *in vivo* validation of TAT-STI1$_{230-245}$ peptide effects on tumor xenografts is the study of peptide bioavailability, tissue distribution and half-life. To observe this and, especially, to confirm that TAT-STI1$_{230-245}$ is diffusible and crosses the cell membrane, we conjugated a dansyl chloride fluorophore (Aarts et al., 2002; Brebner et al., 2005) to the TAT-STI1$_{230-245}$ peptide.

We assessed the U87MG-cell staining pattern of the dansyl-TAT-STI1$_{230-245}$ peptide and the irrelevant dansyl-TAT-STI1$_{422-437}$ peptide, as demonstrated in Figure 10. The peptides were able to cross the cell membrane and they labeled cells efficiently.

STI1/Hop$_{230-245}$ peptide and its TAT-associated forms promote increase in memory formation and can be used as neuroprotective agents against cognitive deficits in patients with brain tumors.

The improvement in treatments of patients with brain cancer has led to a higher survival rate. However, it has also increased cognitive deficits, particularly because of side effects of drugs and radiotherapy. The use of agents designed to protect neurons against apoptosis or neurodegeneration is an interesting approach to be considered (Gehring et al., 2008). Previous studies suggested that STI1$_{230-245}$ peptide was able to promote neuronal survival (Chiarini et al., 2002; Zanata et al., 2002), neuronal differentiation (Lopes et al., 2005) and long-term memory (LTM) consolidation in rats (Coitinho et al., 2007). As demonstrated in the present studies, while inhibiting proliferative activity in tumors derived from glia cells (Figures 7 to 9), the peptides STI1$_{230-245}$ and Hop$_{230-245}$, as well as their counterparts associated to TAT peptide (TAT- STI1$_{230-245}$ and TAT-Hop$_{230-245}$), have a potent effect in memory consolidation in rats (Figure 11) due to their ability to protect neurons.

Therefore, the STI1/Hop$_{230-245}$ peptides can have a dual effect in the treatment of glioblastomas. The first one is to decrease tumor proliferation mediated by secreted STI1/Hop and the second one is its protective effects in neurons and improvement in patients´cognition.

Hop is highly expressed in GBM patients

Additionally, we investigated the Hop gene expression profile from 76 human glioblastoma samples using quantitative RT-PCR. It was observed that glioblastoma multiforme (GBM) expressed higher Hop levels (Figure 12) when compared to normal tissue. These results are particularly significant because they demonstrate the connection between STI1/Hop expression and cancerous states *in vivo*, i.e., in human tissue samples and not just in cancer cell lines growing in culture.

Besides the TAT peptide described above, other molecules that allow drugs to cross the blood-brain barrier (BBB) have been proposed. These molecules can bind to receptors which are responsible for maintaining the integrity of the BBB and brain homeostasis. One important receptor in this

regard is the lipoprotein receptor-related protein (LRP), which possesses the ability to mediate transport of ligands across endothelial cells of the BBB (Shibata et al., 2000, and Ito et al., 2006). The peptide called Angiopep-2 (TFFYGGCRGKRNNFKTEEY), an aprotinine-derived peptide, is able to bind LRP and promote drug delivery in the CNS (Demeule et al., 2008). Angiopep-2 has been recently conjugated to paclitaxel, and improved therapeutic efficacy was observed in orthotopic models of primary and metastatic brain cancer (Régina et al., 2008). Conjugation of $STI1_{230-245}$ and $Hop_{230-245}$ with Angiopep-2 and other peptides involved in transport across the BBB would also provide enhanced bioavailability of the peptides of the present invention to the site(s) of brain cancer in a patient.

It is also known that peptide cyclization can enhance stability without the loss of biological activity. (Pakkala et al., 2007 ; Yokoyama et al., 2004). This approach can be used with $STI1_{230-245}$ and $Hop_{230-245}$ peptides and their conjugates as disclosed herein to enhance their stability.

In addition, we have established a colony of nude (immunosuppressed) mice that is currently under expansion. These animals will be used to perform glioblastoma-cell engraftment and to evaluate the role of $STI1_{230-245}$ and $Hop_{230-245}$ peptides, and their TAT-associated counterparts, on tumor growth and survival. Due to the fact that they are more readily accepted in xenograft models and due to their capacity for tumorigenesis, U87MG cells will be used instead of A172 cells for these studies. It is anticipated that the results from these studies will provide further support for the notion that the inventive peptides have use as agents for the treatment of brain cancers and other cancers linked with the interaction between PrP^C and STI1/Hop.

Our studies showed that: 1) Hop is secreted by a glioblastoma cell line; 2) STI1 induces proliferation of distinct glioma cell lines; 3) the Erk and Akt signaling pathways mediate STI1-induced proliferation; 4) STI1 does not induce proliferation in normal astrocytes; 5) STI1-induced proliferation of A172 cells depends on its PrP^C binding domain; 6) both $STI1_{230-245}$ and $Hop_{230-245}$ peptides inhibit STI1-induced proliferation of A172 and U87MG cells; 7) the TAT-conjugates of $STI1_{230-245}$ and $Hop_{230-245}$ also inhibit glioblastoma cell proliferation; 8) conjugation of the peptides with TAT allows them to cross the cell membrane, most importantly the blood-brain barrier; 9) the $STI1_{230-245}$

peptide and its TAT conjugate enhance cognition *in vivo*; and 10) high expression of the Hop gene is associated with glioblastoma tissue.

Pharmacological blockade of the protein kinases Mek and PI3K abolished activation of Erk and Akt, respectively, and STI1/Hop-induced proliferation of A172 cells. In addition, treatment with STI1/Hop activated Erk and Akt, indicating the involvement of these signaling pathways in the proliferative effect. Treatment with LY294002 induced an increase in the phosphorylation levels of Erk, a finding that suggests the existence of crosstalk between these pathways (Corradetti and Guan 2006). Although activation of MAPK pathways is commonly related to an increase in proliferation, paradoxical effects of MAPK activation regarding its duration and intensity have already been described (Marshall 1995; Sewing, Wiseman et al. 1997; Sebolt-Leopold and Herrera 2004; Murphy and Blenis 2006; Sharrocks 2006). In addition, previous studies showed that Akt activation may cause downregulation of the MAPK pathway (Guan et al. 2000; Moelling et al. 2002; Zimmermann and Moelling 1999).

We showed that STI1/Hop imposes a small and transient activation of Erk, which leads to increased proliferation. On the other hand, when PI3K was inhibited, STI1/Hop-induced activation of Erk was more intense and durable, possibly because in this situation Erk pathway activity was not counterbalanced by Akt. In fact, this pattern of Erk activation may cause cell cycle arrest (Bottazzi et al. 1999; Pumiglia and Decker 1997). Cells subjected to treatment solely with LY294002 or STI1/Hop showed a similar increase in Erk activation as assayed after a 5-minute treatment. However, as opposed to the effect of STI1/Hop treatment on cells, the PI3K inhibitor induced a much more durable Erk activation, which persisted for at least one hour and was not related to increase in proliferation. Together, these data indicate that parallel activation of both the Erk and Akt pathways is required for the proliferative effects of STI1/Hop and that the intensity and duration of Erk activation may ultimately determine the final effect of STI1/Hop on proliferation. A direct involvement of the PKA pathway in the proliferative effect of STI1/Hop is unlikely, because a) upregulation of the cAMP/PKA pathway inhibits proliferation in A172 cells (Chen et al. 1998) and b) our experiments showed that forskolin induces a marked decrease in the incorporation of thymidine.

We showed that distinct tumor-cell lineages respond in distinct ways to STI1 treatment. It is noteworthy that MCF7 cells, the only non-glial tumor cell line tested, in which PTEN function is not disrupted, was not affected by STI1. It is reasonable to conclude that at least part of the distinct STI1 effects on different types of tumor-cell lines are correlated to distinct mechanisms of cell signaling regulation.

The contrast in effect of STI1/Hop upon proliferation in tumor-cell lines and normal glia proliferation is pivotal. These data suggest that drugs capable of disrupting proliferation induced by STI1/Hop would present some kind of selectivity towards tumor cells. In a completely distinct context, Kamal *et al.* (2003) showed that the increased activity of Hsp90, commonly observed in cancers, and probably responsible for an observed tumor response selectivity to the antibiotic geldanamicin, is explained by the formation of multi-chaperone complexes (including STI1/Hop) in tumors but not in normal tissues.

Prions are proteins identified as the etiologic agents of transmissible spongiform encephalopathies, a group of rare neurodegenerative diseases (Prusiner 1998). Although the precise mechanism that leads to the characteristic neurodegeneration observed in these diseases is not fully understood, abundant evidence supports the idea that the expression of cellular prion (PrP^C), the nonpathological isoform of the protein, and its conversion to a pathological conformer, are necessary for development of the disease (Bueler et al. 1993). The neurotoxic property acquired by the pathological isoform compared to the normal protein raised the gain-of-function hypothesis. However, loss of function of the normal PrP^C, caused by its conversion to the pathological isoform, may also contribute to the pathogenesis of prion diseases (Aguzzi and Weissmann 1997; Hetz et al. 2003; Samaia and Brentani 1998). In the last decade, many studies have related PrP^C to distinct physiological functions (Aguzzi and Polymenidou 2004; Martins et al. 2001; Linden et al. 2008).

It has been demonstrated that PrP^C is overexpressed in gastric cancer tissues, and its levels are positively correlated to the process of invasiveness and metastasis (Pan et al. 2006). In gastric cancer cell lines, PrP^C promotes invasion and metastasis through activation of the Mek/Erk pathway and consequent transactivation of MMP11 (Pan et al. 2006). Moreover, PrP^C

ectopic expression promotes tumorigenesis, proliferation and G1/S transition in gastric cancer cells (Liang et al. 2007).

Our data showed that, as opposed to the wild-type recombinant protein, a deletion mutant STI1 which does not bind PrP^C (Lopes et al. 2005) was unable to promote glioma proliferation. These data indicate that endogenous PrP^C is involved in STI1/Hop-induced proliferation of gliomas.

As shown by the disclosure herein, STI1 induces the proliferation of glioma cells but not of normal astrocytes. Furthermore, the PrP^C binding site of STI1/Hop is necessary to achieve this effect. Still further, a mutant STI1 missing the PrP^C binding site fails to induce proliferation.

Our studies have further demonstrated that peptides $STI1_{230-245}$ and $Hop_{230-245}$, and their TAT-conjugates, inhibit the previously demonstrated ability of STI1 to enhance proliferation of glioma cells. The extension of the significance of these effects beyond the context of cell cultures, particularly in connection with the TAT-conjugated peptides, was performed in experiments testing the diffusion and ability of the conjugates to cross the cell membrane. The experiments summarized in Fig. 11 suggested that the peptides did cross the BBB, thus indicating the potential of the peptides in an *in vivo* context.

Further studies on trained rats treated with $STI1_{230-245}$ and TAT-$STI1_{230-245}$ showed that these peptides greatly enhanced long-term memory; animals treated with these peptides showed significantly longer retention latencies than were seen for control animals and those treated with (irrelevant) TAT-$STI1_{61-76}$ peptide.

Accordingly, one aspect of the present invention is a method for treating gliomas which involves interfering with the interaction between STI1/Hop and PrP^C. In one embodiment, this could involve administration of a peptide that mimics the PrP^C binding site of STI1/Hop. Another embodiment would involve reduction of the effective amounts of STI1/Hop, either by a) administering a compound that targets STI1/Hop and interferes with its proliferative function or b) administering a molecule such as an siRNA that interferes with expression of STI1/Hop.

Among the peptides to be used in the practice of the invention are ELGNDAYKKKDFDTAL, ELGNDAYKKKDFDKAL,

YGRKKRRQRRRELGNDAYKKKDFDTAL and YGRKKRRQRRRELGNDAYKKKDFDKAL ($STI1_{230-245}$, $Hop_{230-245}$ and their respective TAT conjugates). However, the invention is by no means limited to these exemplary peptides. For example, additional peptides to be used in the practice of the invention are cyclized versions of the ones recited above. Still further examples are TFFYGGCRGKRNNFKTEEYELGNDAYKKKDFDTAL and TFFYGGCRGKRNNFKTEEYELGNDAYKKKDFDKAL (the $STI1_{230-245}$ and $Hop_{230-245}$ peptides conjugated to Angiopep-2) and cyclized forms thereof. The invention also encompasses functional variants of these peptides and other peptides capable of mimicking the PrP^C binding site of STI1/Hop. By functional variants are meant, for example, derivatives of the peptides, and their cyclized forms, containing one or more amino-acid additions, deletions, insertions or substitutions, or combinations of these changes. The invention is not limited to STI1/Hop peptides and functional variants thereof conjugated to TAT or Angiopep-2. It is expected that the invention can also be practiced with any STI1/Hop conjugate, wherein the peptide conjugated to the STI1/Hop enables crossing of the BBB. The peptides of the invention can be produced by any of the means of synthesis well known to those of skill in the art (see, e.g., Merrifield and Stewart 1965).

Another aspect of the invention concerns antibodies raised against peptides that mimic the PrP^C binding site of STI1/Hop. Such antibodies could be raised against, for example, the peptides disclosed above, as well as against functional variants of these peptides and other peptides capable of mimicking the PrP^C binding site of STI1/Hop. Such antibodies could also be used in the treatment of cancers triggered by the interaction between STI1/Hop and PrP^C. Such antibodies may be produced by any of the techniques well known to one of skill in the art. (See, for example, Monoclonal Antibodies: Methods and Protocols, R. Rose and M. Albitar, Eds., Humana Press, 1st Edition (2007) and Antibodies: A Laboratory Manual, Harlow and Love, Cold Spring Harbor Laboratory Press (2003).) Although monoclonal antibodies are preferred for the practice of the invention, the invention also encompasses polyclonal antibodies of suitable specificity.

The invention is not limited to the treatment of gliomas. It is well known that STI1/Hop is also overexpressed in, for example, the colon and the

stomach. Thus, the methods of the present invention, and the compounds to be used to practice the methods, are applicable also to the treatment of such cancers as colon cancer, colorectal cancer, gastric cancer, glioblastoma, medulloblastoma and astrocytoma.

The invention is not limited to conditions brought about by overexpression of STI1/Hop. As disclosed earlier, it would be expected that the reduction of normal levels of STI1/Hop would lead to the reduction of cancer-cell proliferation and, hence, alleviation of the cancer itself.

Another embodiment of the invention is directed to methods employing the peptides of the present invention for diminishing and even eliminating the side effects of drug therapy and radiotherapy used in treating patients with brain cancers. Such side effects include neuronal death and loss of neuronal differentiation. They further include diminished cognitive function, for example reduced long-term memory consolidation.

Yet another aspect of the invention concerns methods for identifying compounds suitable for the treatment of cancers regulated by the binding of STI1/Hop to PrP^C. Such methods involve monitoring test compounds for their ability to reduce STI1/Hop-PrP^C interaction directly, to reduce the amount of endogenous STI1/Hop that is functional and/or to reduce the amount of STI1/Hop expressed in cancer cells or potentially cancerous cell lines.

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FIGURE LEGENDS

Figure 1: Hop is secreted by A172 human glioblastoma cell line and induces proliferation. ***A.*** Western blot from A172-cell lysate probed against STI1/Hop antibody (Bethyl) reveals a single band at the expected molecular weight (66kDa). As an isotype control, A172-cell lysate was also probed against an irrelevant IgG. ***B.*** A172 cells cultured in serum-free culture media for 24 hr were fixed and immunolabeled with an anti-STI1/Hop antibody (Bethyl) (Left panel). The right panel represents a negative control for the anti-STI1/Hop antibody to exclude unspecific staining from the secondary antibody. Nuclei in both panels were stained with DAPI. Bar, 100µm. ***C.*** Western blot of A172 conditioned media (CM) probed with anti-STI1/Hop (Bethyl) antibody shows a band at the expected molecular weight range. The identity of the band is confirmed by its disappearance after Hop immunodepletion of CM. The pellet resulting from immunodepletion was subjected to a western blot assay that shows Hop (Pellet). CM, integral conditioned media; depleted, immunodepleted CM. The lanes are representative of equivalent starting cell numbers. Lower bands observed in the CM and Pellet lanes correspond to protein degradation and IgG heavy chain respectively. ***D.*** A172 cells were cultured in serum-free media and subjected to STI1 treatment (170nM) for 24 hr. Proliferation was determined as described herein. Values are mean ± standard error; n=15; *$P<0.001$ versus control.

Figure 2: MAPK and PI3K pathways are involved in STI1-induced proliferation of glioma cells. ***A.*** A172 cells were cultured in serum-free media and subjected to distinct treatments for 24 hr. Inhibitors were added 10 minutes before STI1 and abolished the STI1 effect. Proliferation was determined as described herein; n=9. *$P<0.05$ versus control. Values are mean ± standard error. ***B.*** Trypan blue cell viability assay. A172 cells were cultured in serum-free media, subjected to distinct treatments for 24 hr and assayed for viability. Results are expressed as the percentage of dead cells in different experimental groups; n=9. Values are mean ± standard error.

C. A172 cells were subjected to STI1 treatment for 5 minutes. ***D.*** Densitometry of phospho-Erk western blots as shown in Fig. 2 C; n=3. Values are mean ± standard error, normalized to untreated cells. ***E.*** A172 cells were subjected to STI1 treatment for 1 minute. ***F.*** Densitometry of phospho-Akt western blots as shown in Fig. 2 E; n=3. Values are mean ± standard error, normalized to untreated cells. ***G.*** A172 cells were subjected to distinct treatments for the indicated times. ***H.*** Densitometry of phospho-Erk western blots as shown in Fig. 2 G; n=3. Values are mean ± standard error, normalized to untreated cells. In co-treatments, inhibitors were added 10 minutes prior to STI1. ***I.*** A172 cells were cultured in serum-free media and subjected to forskolin treatment for 24 hr. Proliferation was determined as described herein. n=9. *P<0.001 versus control. Values are mean ± standard error.

Figure 3: STI1 induces proliferation in distinct glioma cell lines. Cells were cultured in serum-free media and subjected to STI1 treatment for 24 hr. Proliferation was determined by quantitative measurement of [^{3}H]-thymidine incorporation (6.7uCi/ml, 6-hour pulse). The results are respectively normalized to the rate of proliferation (100%) in serum-free media. (CTR); n=9; C6, rat glioma; MCF7, human breast adenocarcinoma; U87, human glioblastoma. *P<0.01 versus control. Values are mean ± standard error.

Figure 4: STI1 does not induce proliferation in normal glia. Astrocytes obtained from neonate rats were cultured in serum-free media ***(A)*** or in 5% fetal bovine serum (FCS) (***B***) and subjected to STI1 treatment for 24 hr. ***C.*** FCS 5% effect upon astrocyte proliferation. Proliferation was determined as described herein. n=15; *P<0.05 versus control; **P<0.001 versus control. Values are mean ± standard error. ***D.*** Astrocytes cultured in serum-free media for 48 hr were fixed and immunolabeled for GFAP (Left panel). The right panel represents a negative control for the anti-GFAP antibody to exclude unspecific staining from the secondary antibody. Nuclei in both panels were stained with DAPI. Bar, 50µm.

Figure 5: STI1-induced proliferation depends on its PrP^C binding site. ***A.*** STI1$\Delta_{230\text{-}245}$ does not promote proliferation. Cells were cultured in serum-

free media and subjected to wild-type (STI1) or mutant ($STI1\Delta_{230-245}$) treatment at distinct concentrations for 24 hr. Proliferation was determined as described herein. n=9; *P<0.001 versus control. Values are mean ± standard error. ***B.*** Cells were incubated with an anti-PrP^C antibody raised in *Prnp-null* mice or with an irrelevant mouse IgG for negative control. Flow cytometry assay shows PrP^C expression at the cell surface (anti-PrP^C) as compared to negative control (irrel).

Figure 6: $STI1_{230-245}$ peptide that represents the binding site at the PrP^C molecule is unable to promote proliferation in A172 glioblastoma cell line. Cells were cultured in serum-free media and subjected to $STI1_{230-245}$ (ELGNDAYKKKDFDKAL) or $STI1/Hop_{61-76}$ (GCKTVDLKPDWGKGYS) peptide treatment at the indicated concentration for 24 hr. Proliferation was determined by quantitative measurement of [^{3}H]-thymidine incorporation (6.7uCi/ml, 6-hour pulse). The results are respectively normalized to the rate of proliferation (100%) in serum-free media. (CTR); n=4. Values are means ± standard error.

Figure 7A: Treatment of A172 cells with $STI1_{230-245}$ peptide abrogates cell proliferation mediated by STI1. A172 human glioblastoma cells were plated at $1x10^4$ confluence on glass cover slips of 12mm. After overnight adherence, cells were starved on serum-free media for 30 hours. Cells were subjected to distinct treatments (see picture), for 18 hours, and a BrdU pulse of 32μM was performed on the last 30 minutes of treatment. Immunofluorescence and cell imaging were done in order to permit absolute cell counting. Values represent percentage of BrdU positive cell nuclei from total number of cell nuclei (DAPI staining) on at least four different images of each condition. FCS (fetal calf serum), STI1 (170nM) and/or $STI1_{230-245}$ peptide (170nM) or $STI1/Hop_{61-76}$ (irrelevant N-terminus STI1/Hop peptide, 170nM). *Statistically significant from control (without treatment), p<0.05. Values Bars shown as mean values ± SEM.

Figure 7B: Treatment of U87MG cells with $STI1_{230-245}$ peptide abrogates cell proliferation mediated by STI1. U87MG human glioblastoma cells were plated at $1.5x10^4$ confluence on glass cover slips of 12mm. After overnight adherence, cells were starved on serum-free media for 48 hours. Cells were subjected to distinct treatments (see picture) for 24 hours, and a BrdU pulse of 32μM was performed on the last 2 hours of treatment. Immunofluorescence and cell imaging were done in order to permit absolute cell counting. Values represent percentage of BrdU positive cell nuclei from total number of cell nuclei. FCS (fetal calf serum), STI1/Hop (0.17μM or 8μM) and/or $STI1_{230-245}$ (0.17μM or 8μM) and/or STI1/Hop_{61-76} (irrelevant N-terminus STI1/Hop peptide, **0**.17μM or 8μM). *Statistically significant from control (without treatment), $p<0.001$. Bars shown as mean ± SEM.

Figure 8: $Hop_{230-245}$ peptide inhibits the U87MG cell proliferation mediated by STI1. U87MG human glioblastoma cells were plated at $1.5x10^4$ confluence on glass cover slips of 12mm. After overnight adherence, cells were starved on serum-free media for 48 hours. Cells were subjected to distinct treatments (see picture) for 24 hours, and a BrdU pulse of 32μM was performed on the last 2 hours of treatment. Immunofluorescence and cell imaging were done in order to permit absolute cell counting. Values represent percentage of BrdU positive cell nuclei from total number of cell nuclei. STI1 (0.17μM) and/or $Hop_{230-245}$ (0.17μM or 8μM) and/or STI1/Hop_{61-76} (irrelevant STI1/Hop_{61-76}, 0.17μM or 8μM). *Statistically significant from control (without treatment), $p<0.01$. Bars shown as mean ± SEM.

Figure 9: Treatment of U87MG cells with TAT-$STI1_{230-245}$ and TAT-$Hop_{230-245}$ peptides inhibits cell proliferation mediated by STI1. U87MG human glioblastoma cells were plated at $1.5x10^4$ confluence on glass cover slips of 12mm. After overnight adherence, cells were starved on serum-free media for 48 hours. Cells were subjected to distinct treatments (see picture) for 24 hours, and a BrdU pulse of 32μM was performed on the last 2 hours of treatment. Immunofluorescence and cell imaging were done in order to permit absolute cell counting. STI1 (0.17μM) and/or TAT-$STI1_{230-245}$ (0.17μM) and/or

TAT-Hop$_{230-245}$ (0.17μM). Values represent percentage of BrdU positive cell nuclei from total number of cell nuclei. *Statistically significant from control (without treatment), p<0.01. Dunnets test. Bars shown as mean ± SEM.

Figure 10: Dansyl TAT-STI1$_{230-145}$ peptide effectively labeled U87MG cells. Images exhibit fluorescence microscope imaging of U87MG cells that received a 2-hour treatment of Dansyl TAT-STI1$_{230-245}$ (left panel) and Dansyl TAT-STI1$_{422-437}$ irrelevant (right panel) peptides. Insets show negative controls.

Figure 11: STI1$_{230–245}$ peptide and TAT-STI1$_{230–245}$ peptides are capable of increasing long-term memory. The latency to step down before treated rats was recorded (Training) and immediately after training animals received a bilateral hippocampal infusion of saline, STI1$_{230–245}$ peptide, TAT-STI1$_{230–245}$ or TAT-STI1$_{61-76}$ irrelevant peptide at the concentration of 15 ng/μl in a total volume of 0.5 μl/side. The latency to step down was tested again 24 hours later (LTM test) and measures Long-term memory (LTM). Data are shown as mean ± SE of step-down latencies (n=12 rats in each group). *p<0.05 vs control.

Figure 12: Hop expression in glioblastomas and normal tissue. The mRNA was extracted from normal brain and glioblastomas tissues and RT-PCR was performed to produce cDNA. Total cDNA obtained from 17 normal brain tissues and 76 glioblastoma samples were evaluated for Hop relative expression in both tissues using Real time-PCR. *Statistically significant from normal tissues, p<0.05. Bars represent mean values.

We claim:

1. A method for the treatment of cancer, which comprises administering to a patient in need thereof an effective amount of a peptide that inhibits the interaction between PrP^C and Hop, thus inhibiting the Hop-induced proliferation of the cancer cells.

2. The method according to claim 1, wherein the cancer is selected from glioma, glioblastoma, medulloblastoma, astrocytoma, colon cancer, colorectal cancer and gastric cancer.

3. The method according to claim 2, wherein the cancer is a glioma.

4. The method according to any one of claims 1-3, wherein the peptide is selected from the group consisting of ELGNDAYKKKDFDKAL, ELGNDAYKKKDFDTAL, YGRKKRRQRRRELGNDAYKKKDFDTAL, YGRKKRRQRRRELGNDAYKKKDFDKAL, TFFYGGCRGKRNNFKTEEYELGNDAYKKKDFDTAL, TFFYGGCRGKRNNFKTEEYELGNDAYKKKDFDKAL, a cyclized form of any one of the peptides or a functional derivative of any one of the peptides or cyclized forms thereof.

5. A method for the treatment of cancer, which comprises administering to a patient in need thereof an effective amount of a compound that targets endogenous Hop or reduces expression of Hop.

6. The method according to claim 5, wherein the cancer is a glioma.

7. A peptide selected from the group consisting of ELGNDAYKKKDFDTAL, YGRKKRRQRRRELGNDAYKKKDFDTAL, YGRKKRRQRRRELGNDAYKKKDFDKAL, TFFYGGCRGKRNNFKTEEYELGNDAYKKKDFDTAL, TFFYGGCRGKRNNFKTEEYELGNDAYKKKDFDKAL, a cyclized form of any one of the peptides or a functional derivative of any one of the peptides or cyclized forms thereof.

8. An antibody raised against a peptide selected from the group consisting of ELGNDAYKKKDFDKAL, ELGNDAYKKKDFDTAL, YGRKKRRQRRRELGNDAYKKKDFDTAL, YGRKKRRQRRRELGNDAYKKKDFDKAL, TFFYGGCRGKRNNFKTEEYELGNDAYKKKDFDTAL, TFFYGGCRGKRNNFKTEEYELGNDAYKKKDFDKAL, a cyclized form of any one of the peptides or a functional derivative of any one of the peptides or cyclized forms thereof.

9. A method for the treatment of cancer, which comprises administering to a patient in need thereof an effective amount of an antibody according to claim 8.

10. The method according to claim 9, wherein the cancer is a glioma.

11. A method for alleviating or eliminating the side effects of drug therapy and radiotherapy used in treating patients with brain cancers, which comprises administering to such a patient an effective amount of a peptide that inhibits the interaction between PrP^C and Hop.

12. The method according to claim 11, wherein the cancer is selected from glioma, glioblastoma, medulloblastoma and astrocytoma.

13. The method according to claim 12, wherein the cancer is a glioma.

14. The method according to any one of claims 11-13, wherein the peptide is selected from the group consisting of ELGNDAYKKKDFDKAL, ELGNDAYKKKDFDTAL, YGRKKRRQRRRELGNDAYKKKDFDTAL, YGRKKRRQRRRELGNDAYKKKDFDKAL, TFFYGGCRGKRNNFKTEEYELGNDAYKKKDFDTAL, TFFYGGCRGKRNNFKTEEYELGNDAYKKKDFDKAL, a cyclized form of any one of the peptides or a functional derivative of any one of the peptides or cyclized forms thereof.

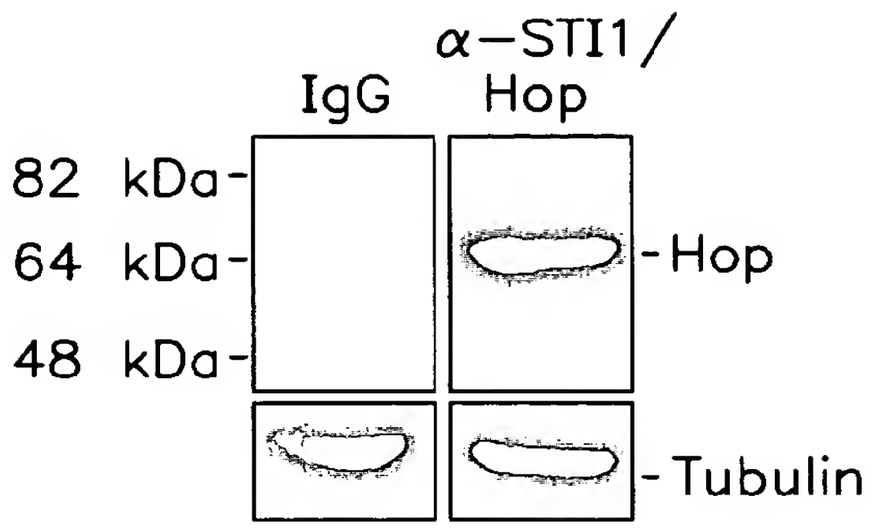

FIG. 1A

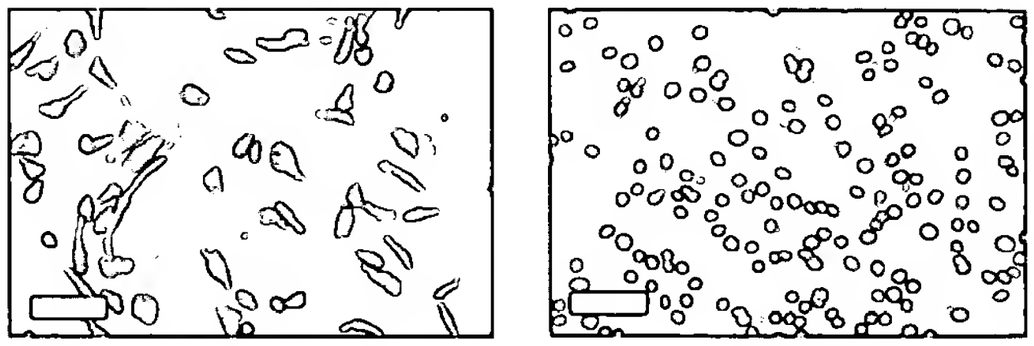

FIG. 1B

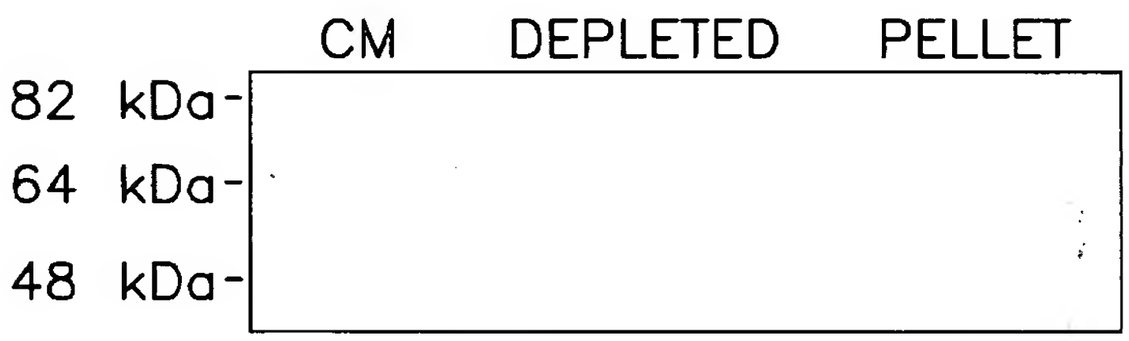

FIG. 1C

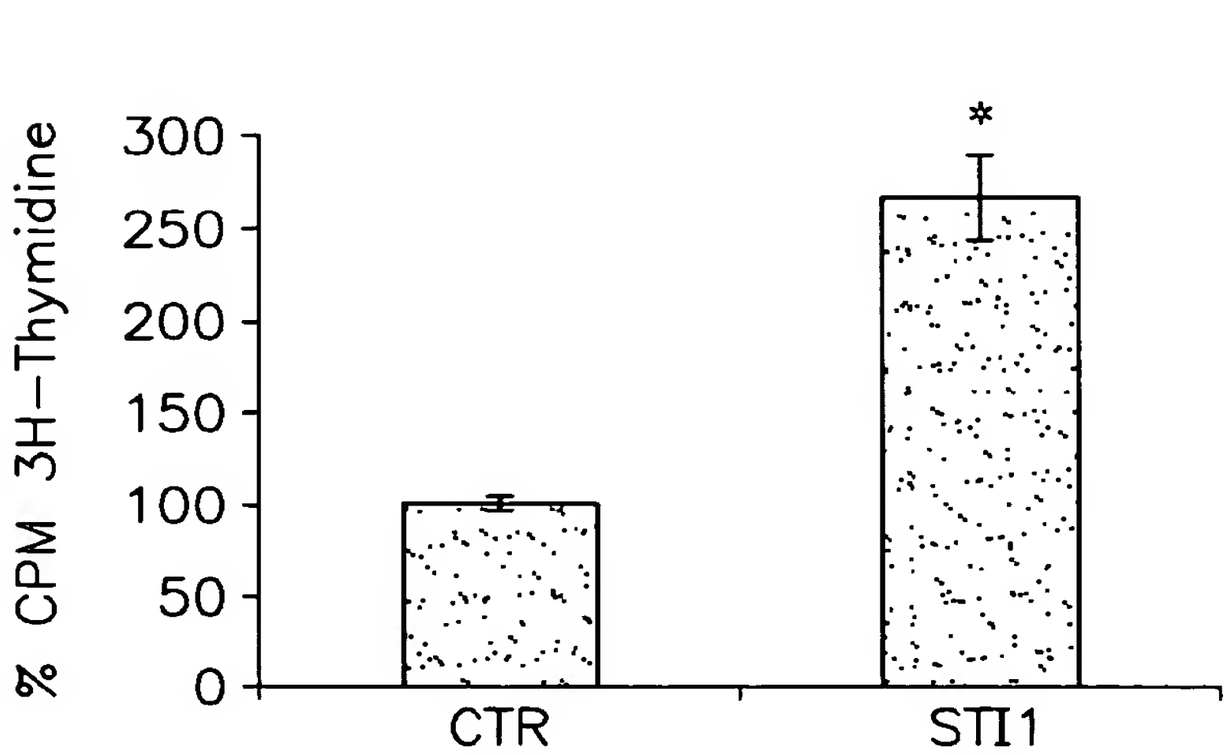

FIG. 1D

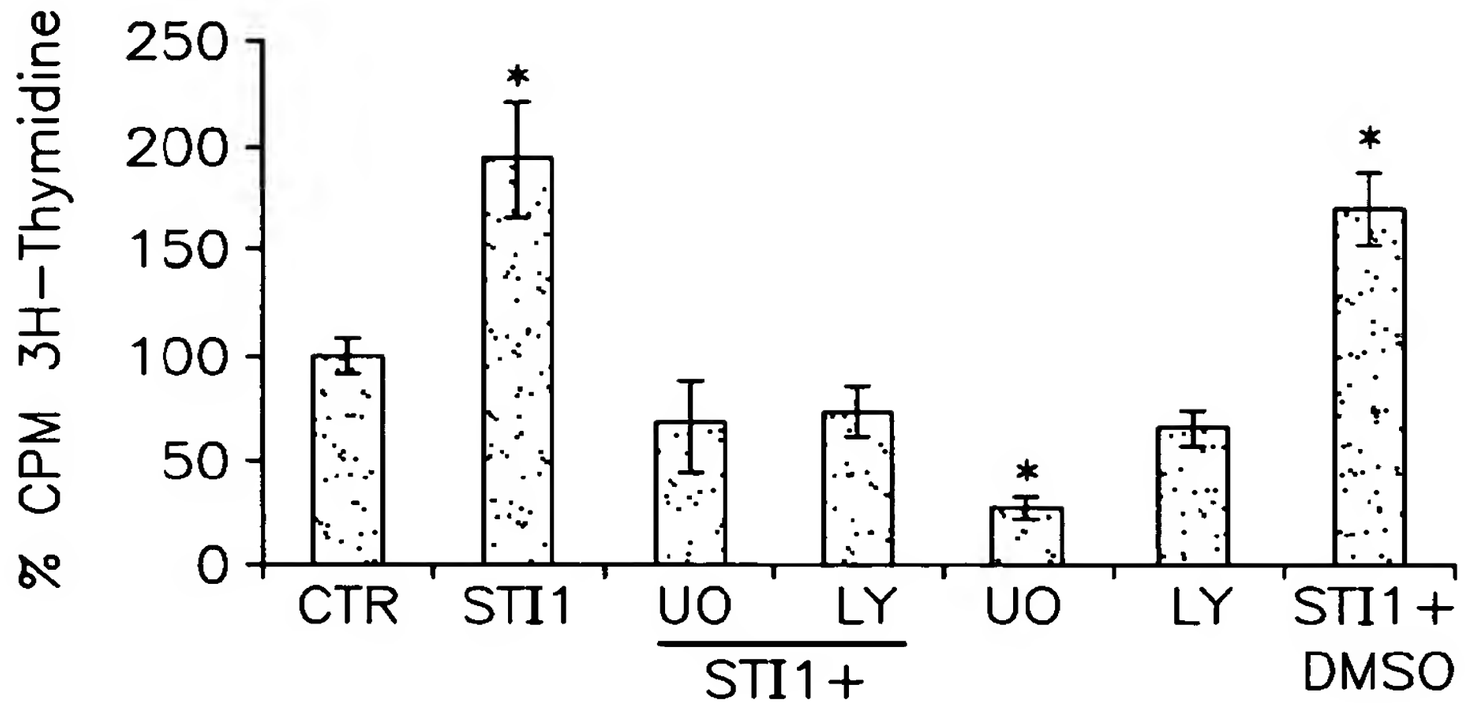

FIG. 2A

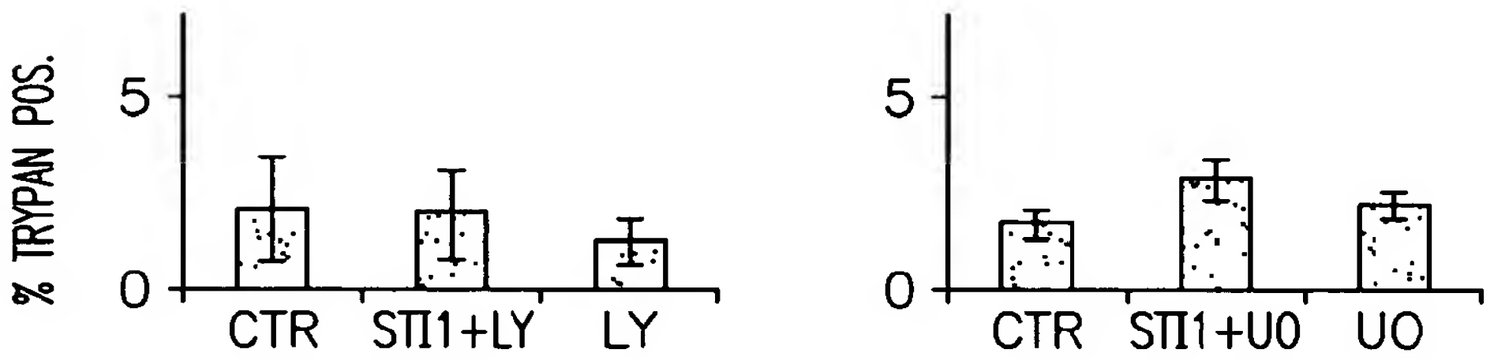

FIG. 2B

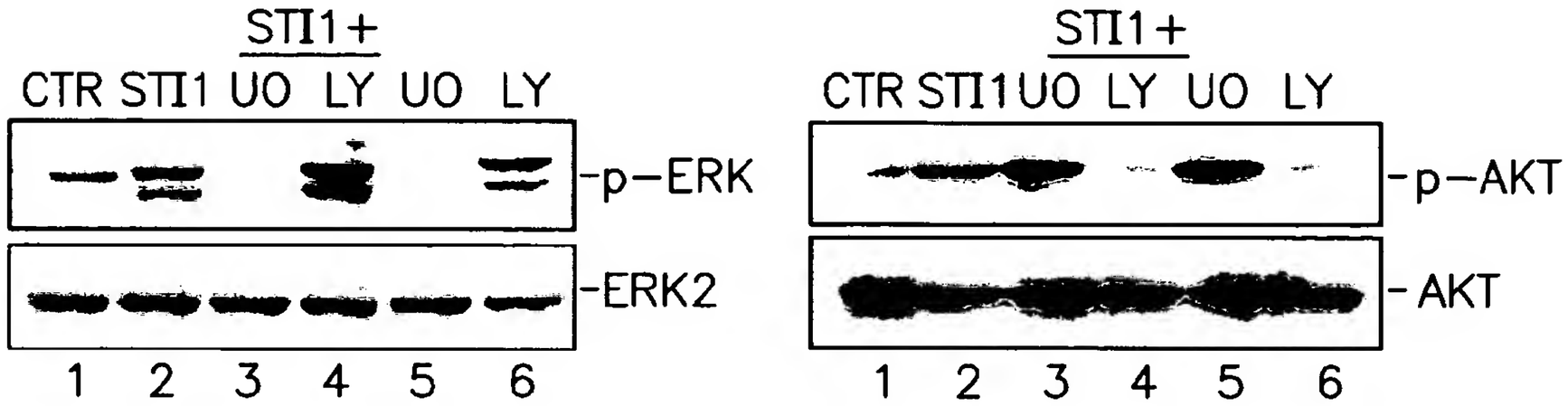

FIG. 2C

FIG. 2E

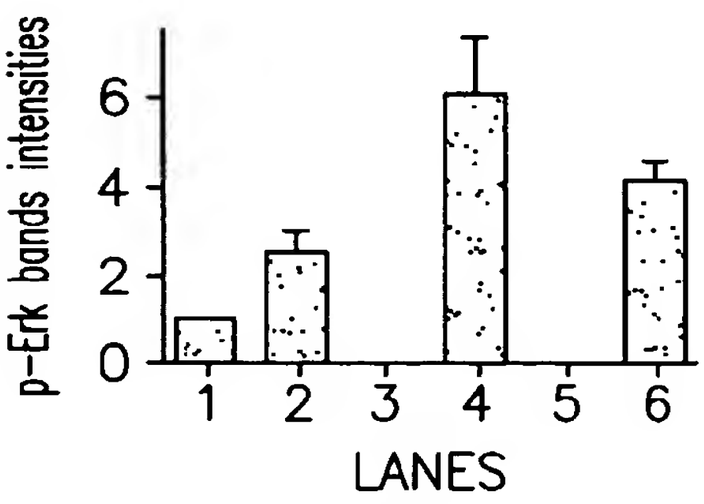

FIG. 2D

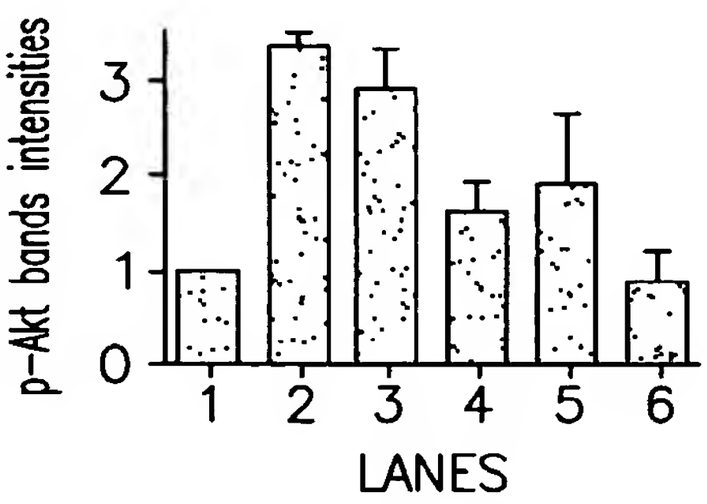

FIG. 2F

Figure 2

(continued)

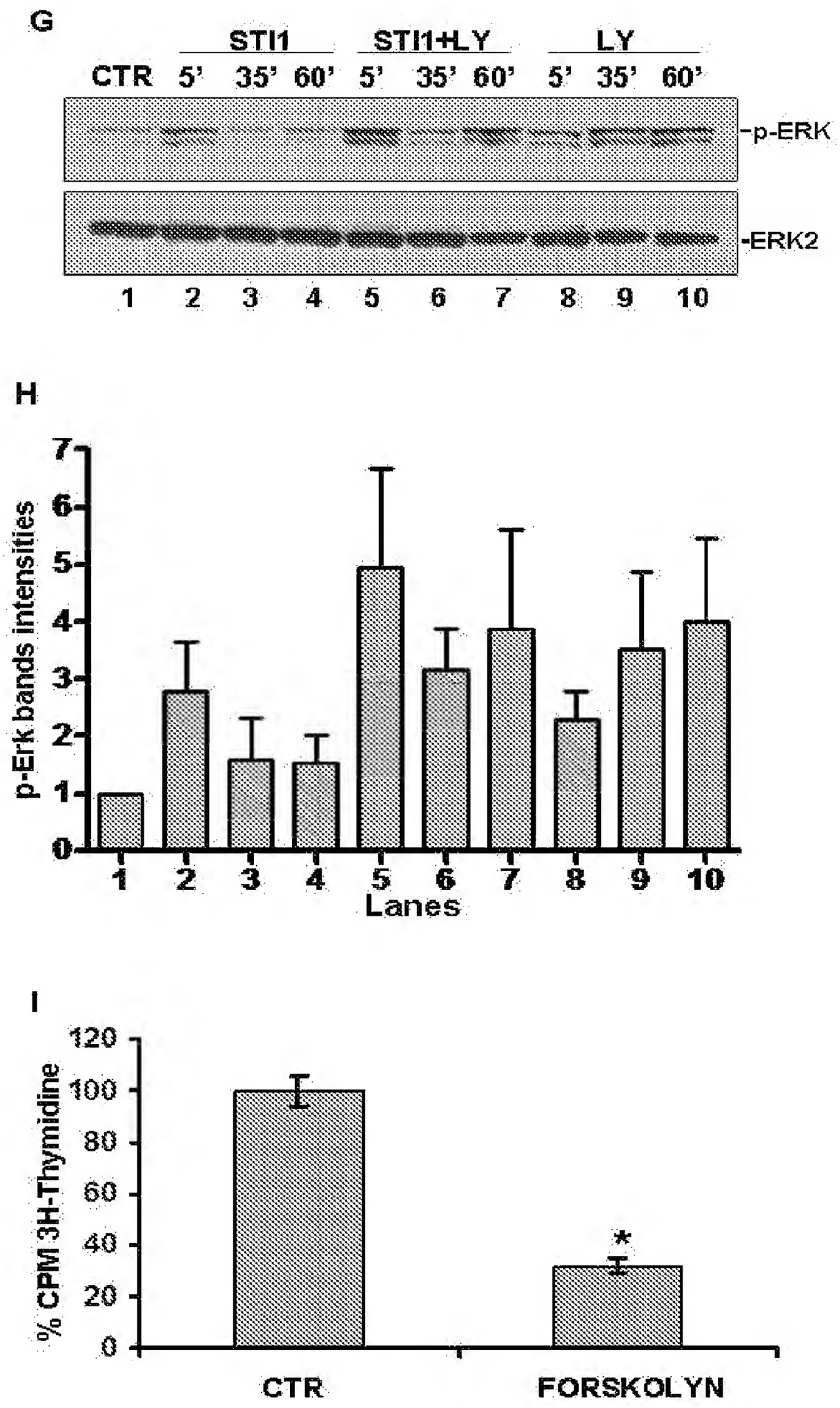

Figure 3

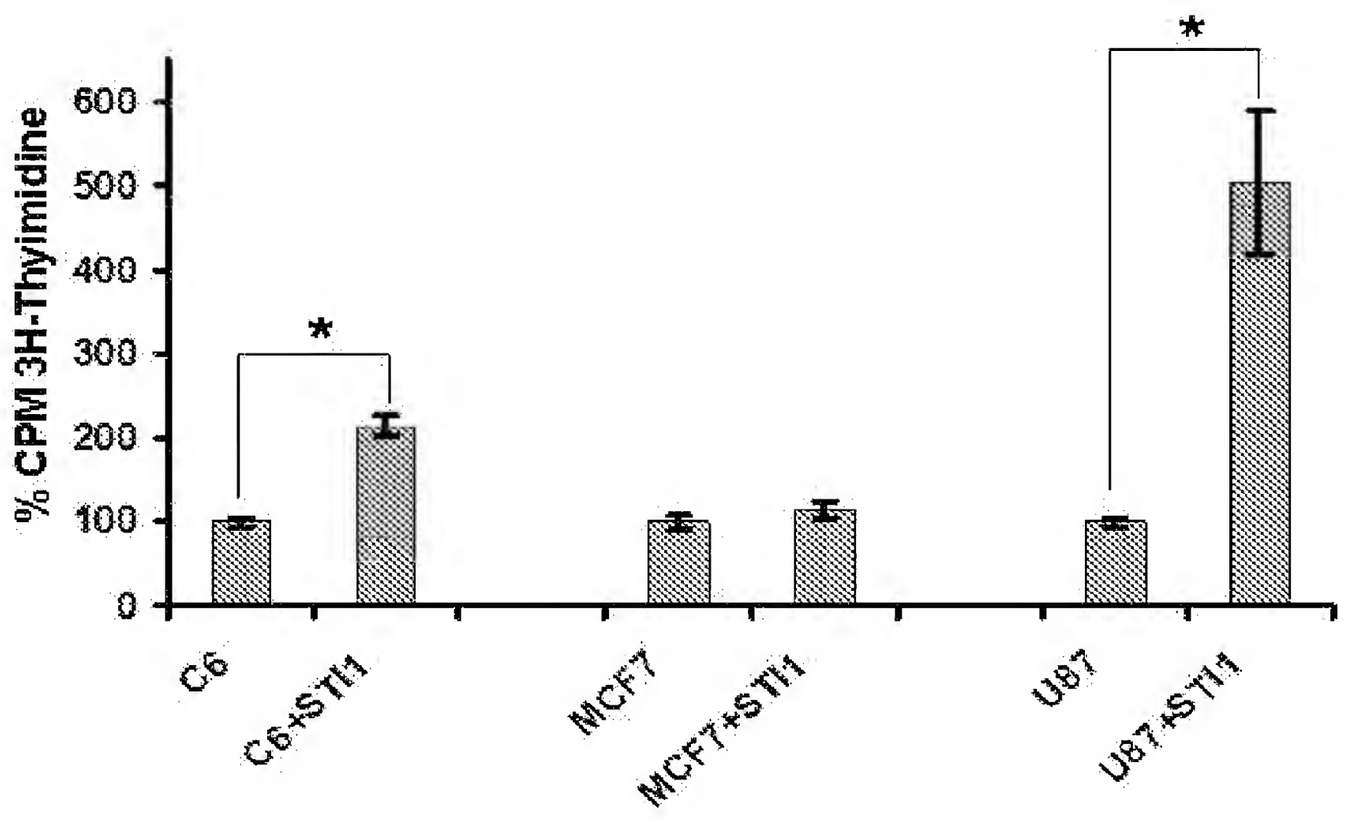
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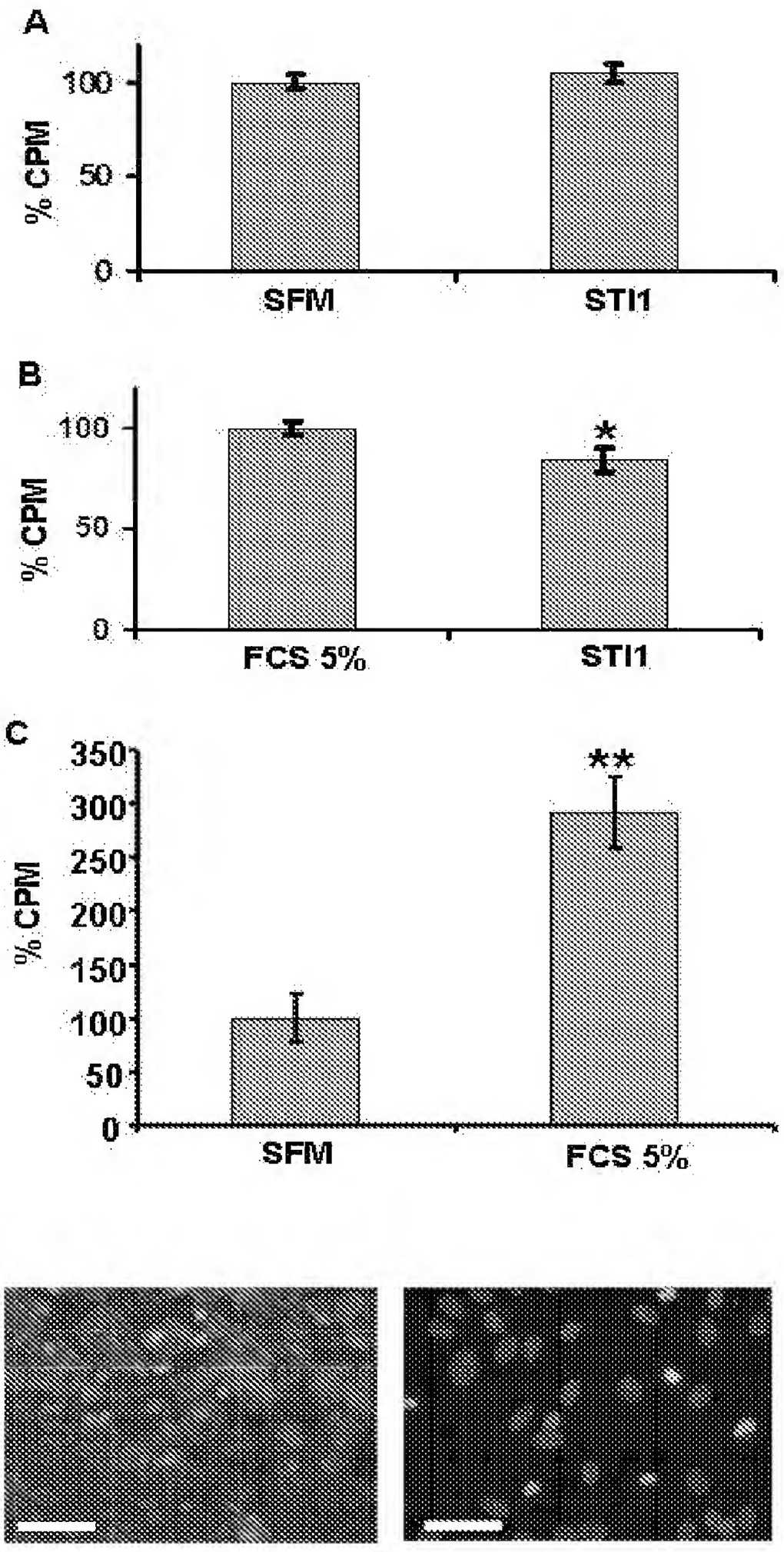
Figure 4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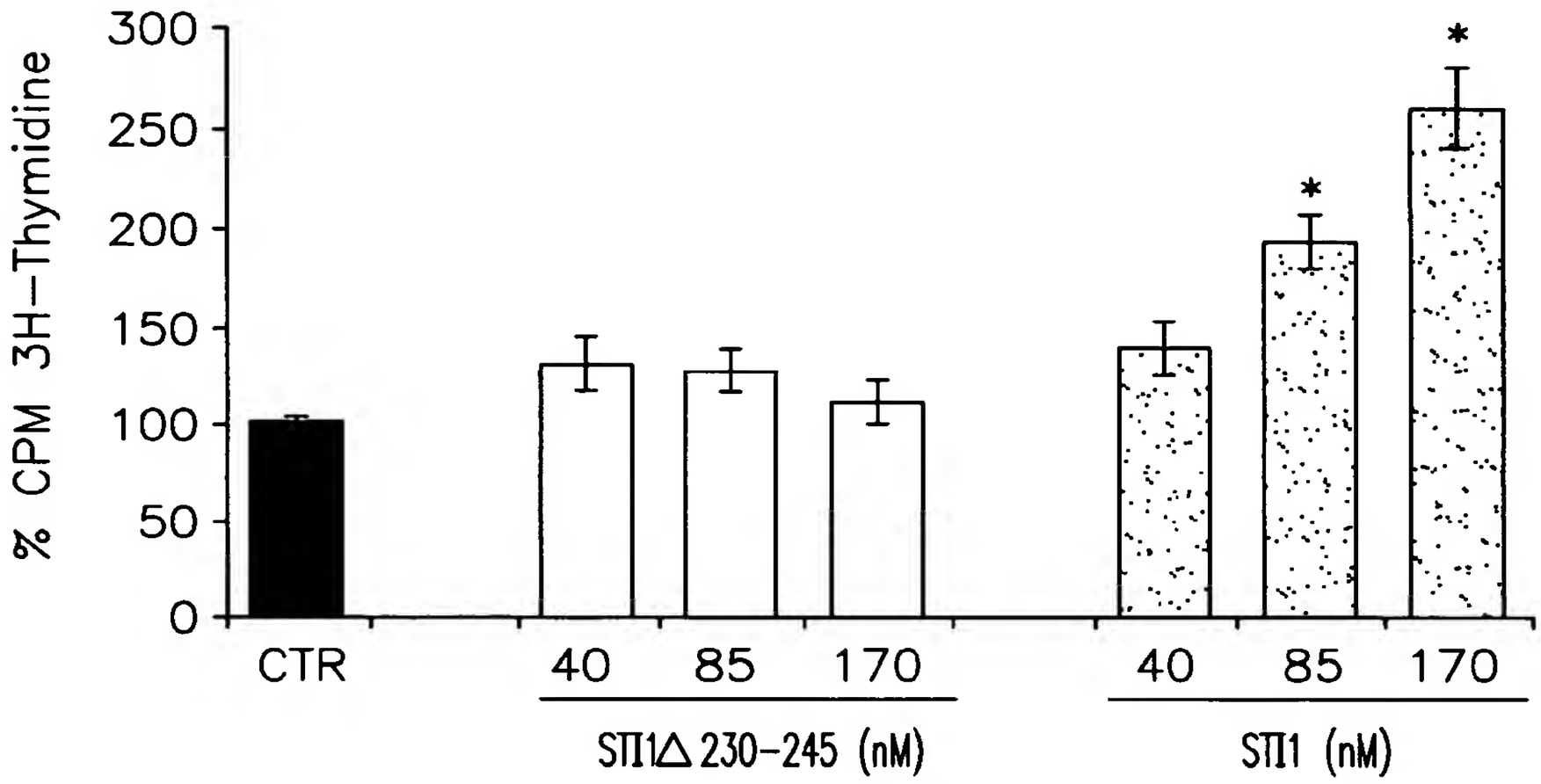
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*

FIG. 5A

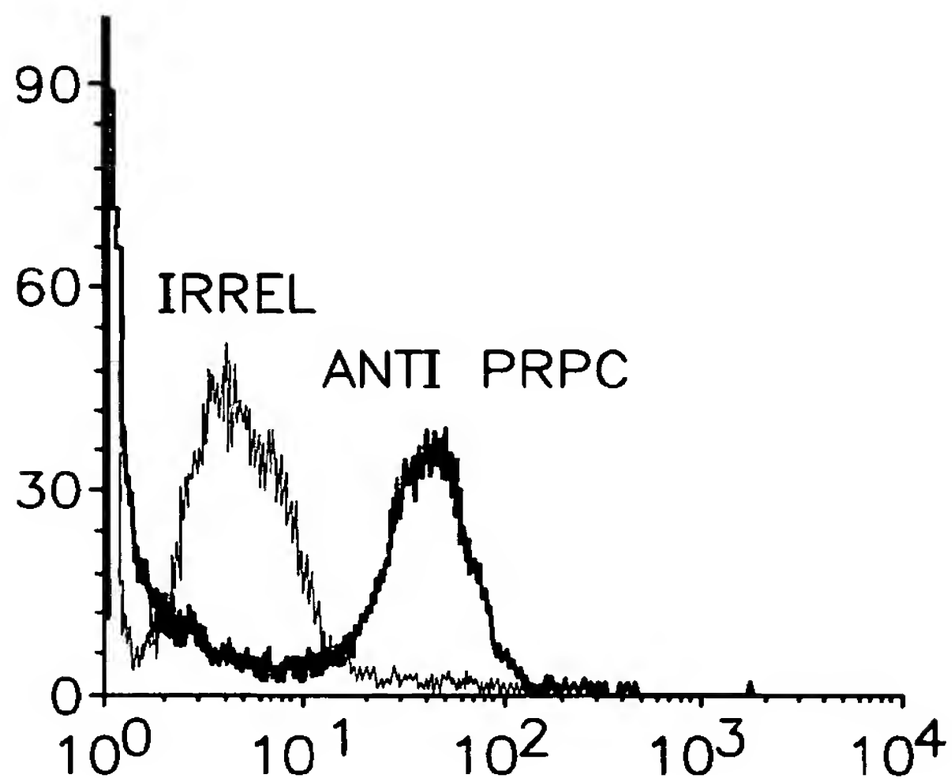

90
60
30
0
IRREL
ANTI PRPC
10^0
10^1
10^2
10^3
10^4

FIG. 5B

Figure 6

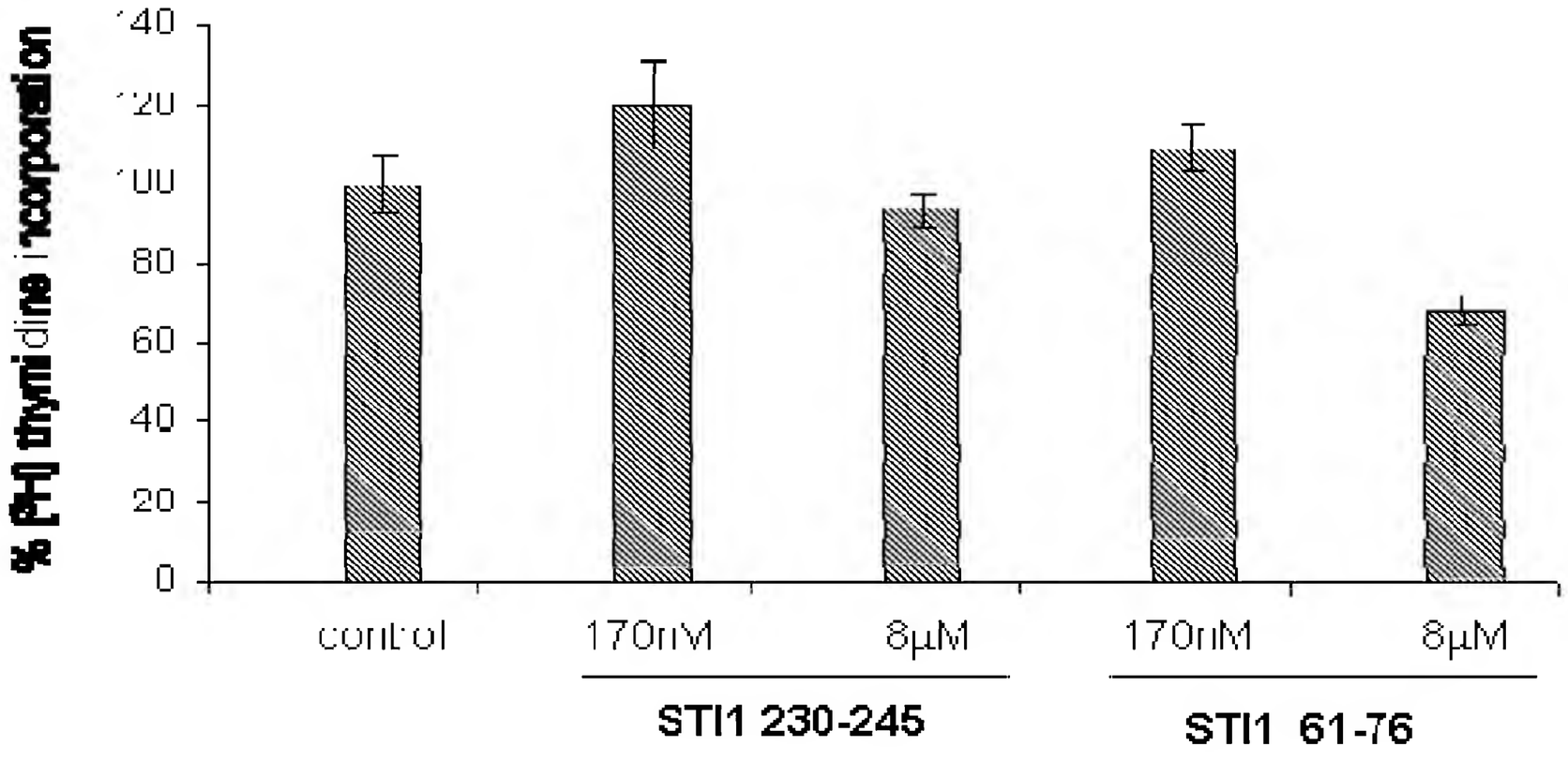

% [3H] thymidine incorporation
140
120
100
80
60
40
20
0
control
170nM
8µM
170nM
8µM
STI1 230-245
STI1 61-76

Figure 7A

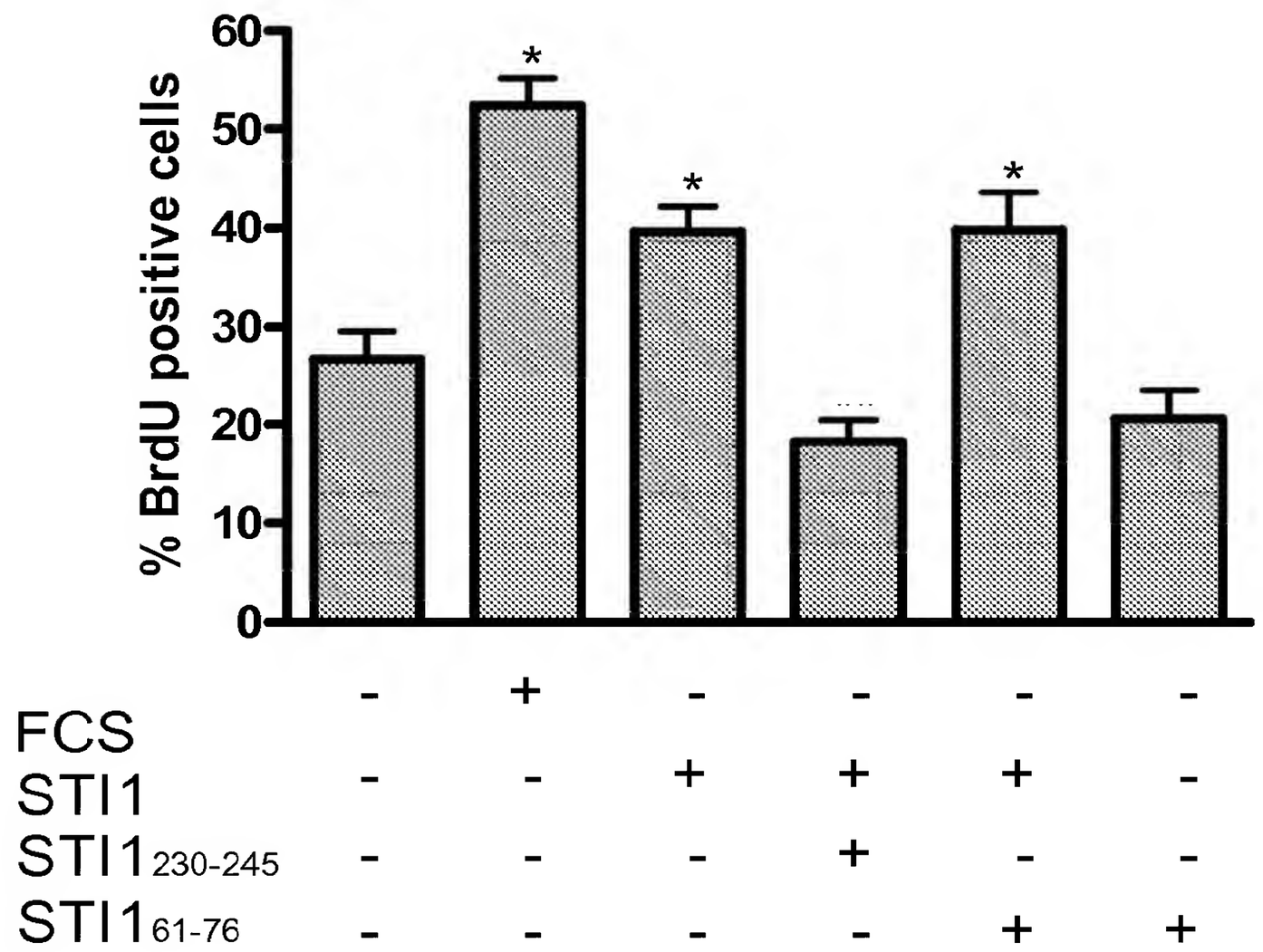

% BrdU positive cells
60
50
40
30
20
10
0
*
*
*
FCS - + - - - -
STI1 - - + + + -
STI1230-245 - - - + - -
STI161-76 - - - - + +

Figure 7B

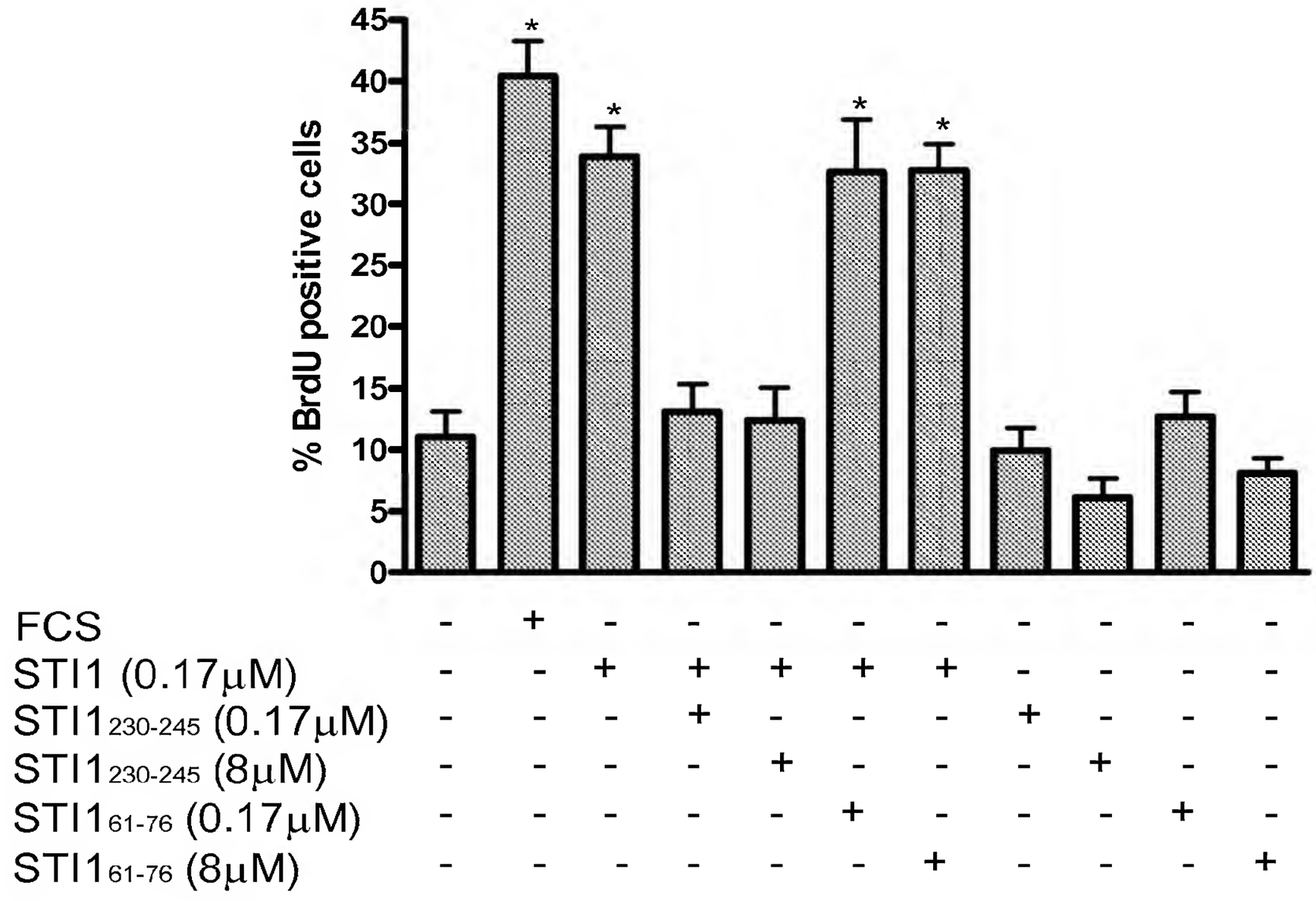

% BrdU positive cells
0
5
10
15
20
25
30
35
40
45
*
*
*
*
FCS - + - - - - - - - - -
STI1 (0.17μM) - - + + + + + - - - -
STI1230-245 (0.17μM) - - - + - - - + - - -
STI1230-245 (8μM) - - - - + - - - + - -
STI161-76 (0.17μM) - - - - - + - - - + -
STI161-76 (8μM) - - - - - - + - - - +

Figure 8

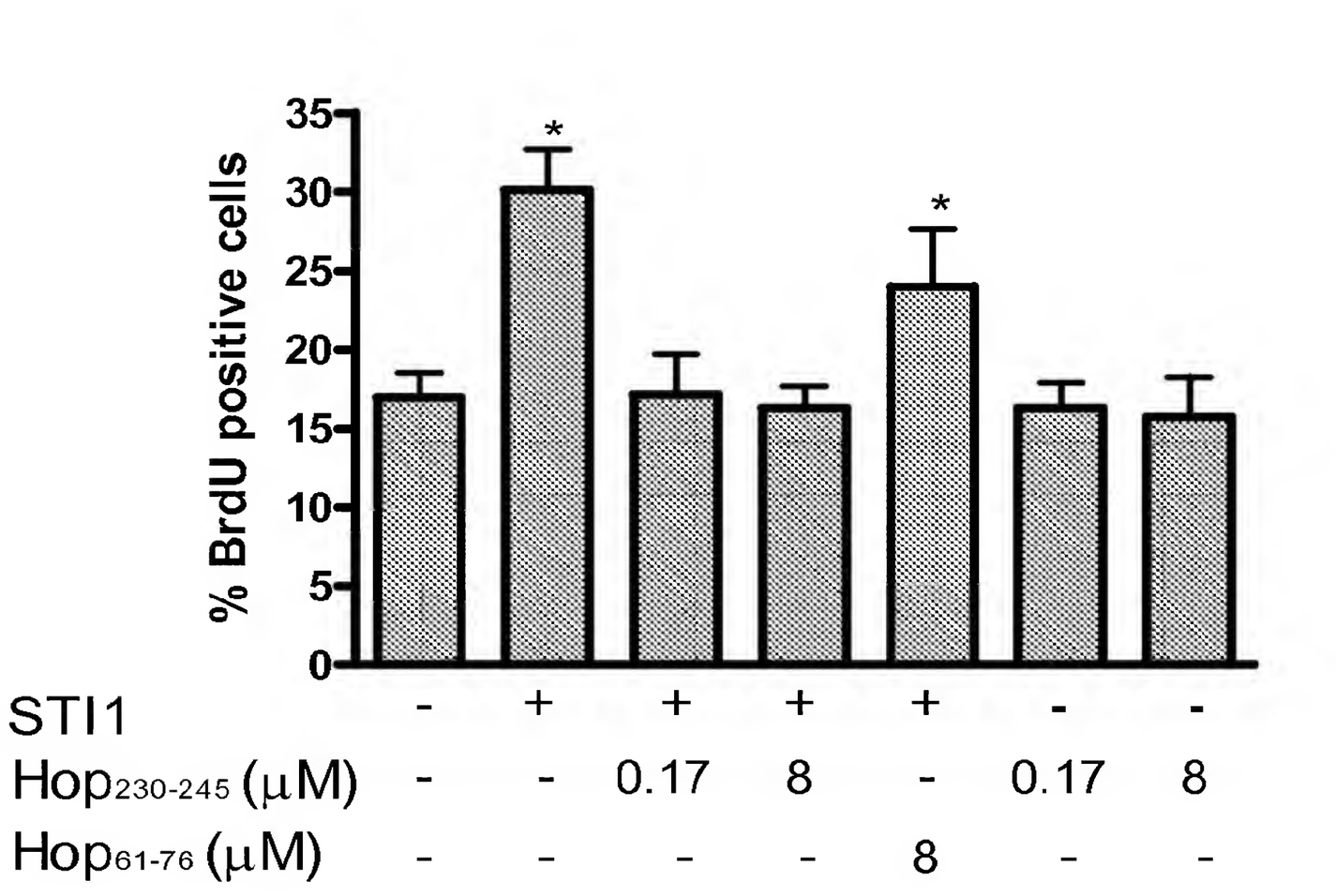

% BrdU positive cells
0
5
10
15
20
25
30
35
*
*
STI1 - + + + + - -
Hop230-245 (μM) - - 0.17 8 - 0.17 8
Hop61-76 (μM) - - - - 8 - -

Figure 9

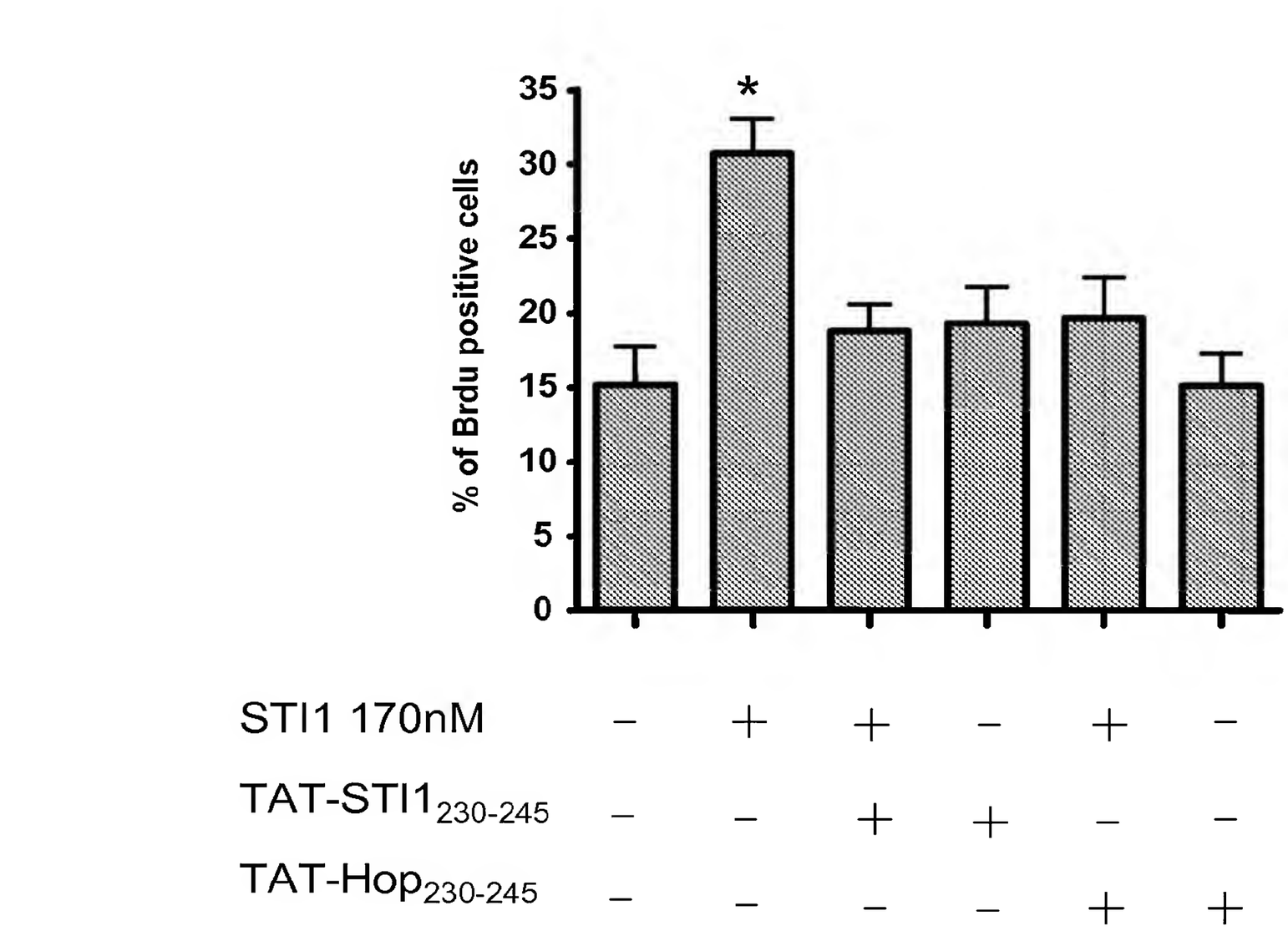

35
30
25
20
15
10
5
0
% of Brdu positive cells
*
STI1 170nM – + + – + –
TAT-STI1230-245 – – + + – –
TAT-Hop230-245 – – – – + +

Figure 10

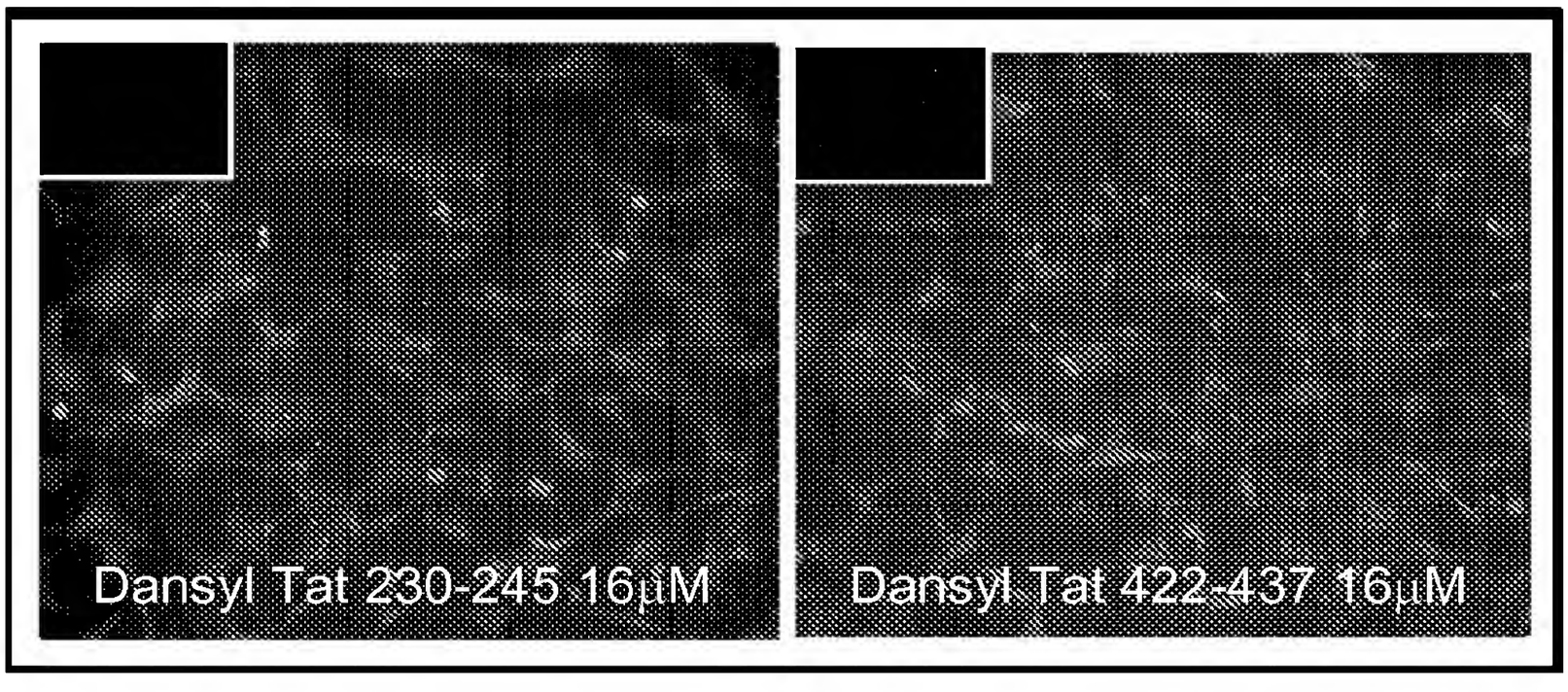


Figure 11

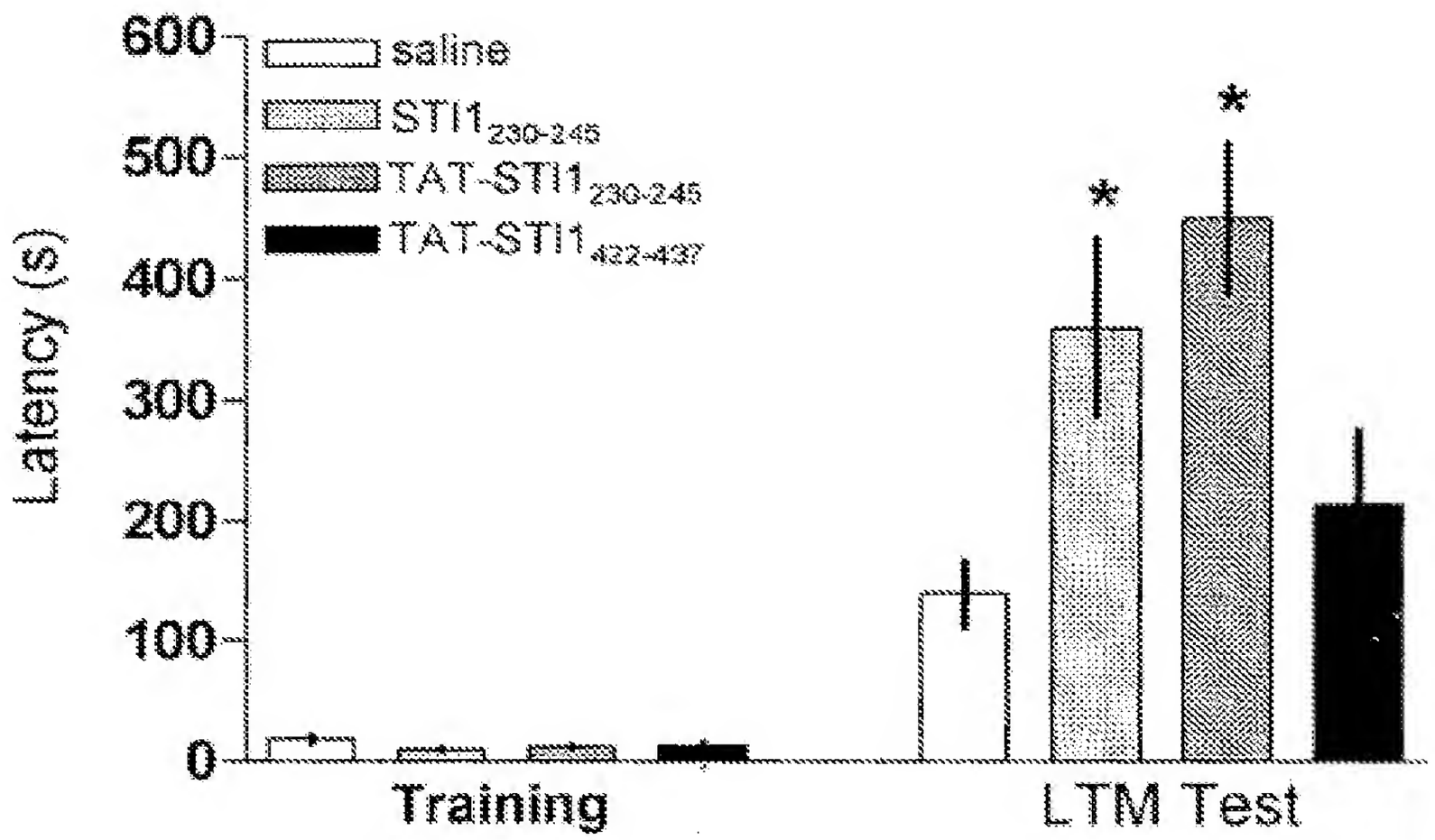
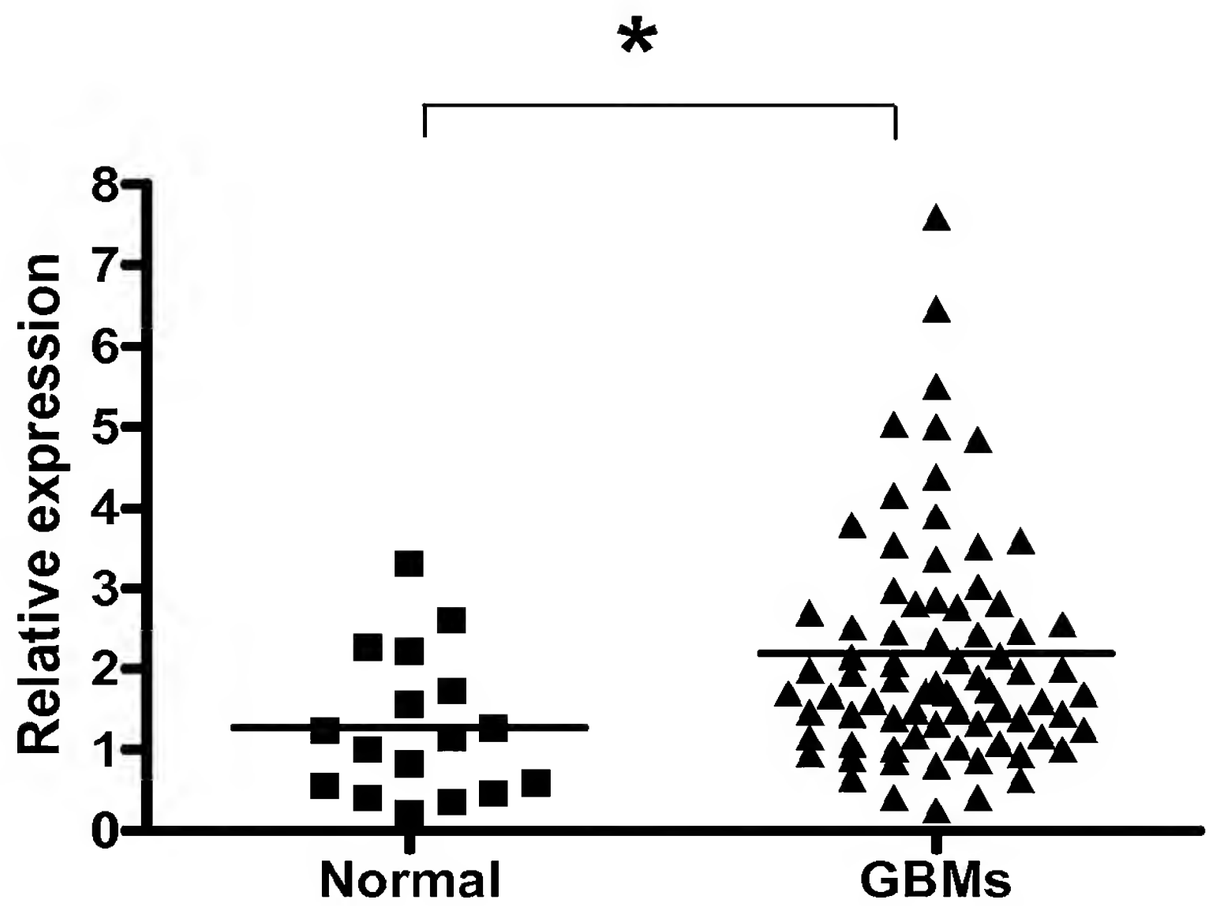

600
500
400
300
200
100
0
Latency (s)
saline
STI1230-245
TAT-STI1230-245
TAT-STI1422-437
*
*
Training
LTM Test

Figure 12



INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/076699

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/04 A61K38/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, EMBASE, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	ERLICH RAFAEL B ET AL: "STI1 promotes glioma proliferation through MAPK and PI3K pathways." GLIA DEC 2007, vol. 55, no. 16, December 2007 (2007-12), pages 1690-1698, XP002510160 ISSN: 0894-1491 cited in the application the whole document	1-14

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[X] Further documents are listed in the continuation of Box C.

[ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
14 January 2009	26/01/2009

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Schnack, Anne

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/076699

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	AMERICO ET AL: "Signaling induced by hop/STI-1 depends on endocytosis" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, ACADEMIC PRESS INC. ORLANDO, FL, US, vol. 358, no. 2, 19 May 2007 (2007-05-19), pages 620-625, XP022083679 ISSN: 0006-291X cited in the application abstract page 620, paragraph 1 - page 621, paragraph 4	1-6,8-14
Y	ZANATA SILVIO M ET AL: "Stress-inducible protein 1 is a cell surface ligand for cellular prion that triggers neuroprotection." THE EMBO JOURNAL 1 JUL 2002, vol. 21, no. 13, 1 July 2002 (2002-07-01), pages 3307-3316, XP002510161 ISSN: 0261-4189 cited in the application abstract page 3315, column 1, paragraph 5	1-6,8-14
A	LOPES MARILENE H ET AL: "Interaction of cellular prion and stress-inducible protein 1 promotes neuritogenesis and neuroprotection by distinct signaling pathways." THE JOURNAL OF NEUROSCIENCE : THE OFFICIAL JOURNAL OF THE SOCIETY FOR NEUROSCIENCE 7 DEC 2005, vol. 25, no. 49, 7 December 2005 (2005-12-07), pages 11330-11339, XP002510162 ISSN: 1529-2401 cited in the application abstract	1-14
A	BLATCH G L ET AL: "Isolation of a mouse cDNA encoding mSTI1, a stress-inducible protein containing the TPR motif" GENE, ELSEVIER, AMSTERDAM, NL, vol. 194, no. 2, 31 July 1997 (1997-07-31), pages 277-282, XP004093355 ISSN: 0378-1119 cited in the application abstract	1-14

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/076699

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HONORÉ B ET AL: "Molecular cloning and expression of a transformation-sensitive human protein containing the TPR motif and sharing identity to the stress-inducible yeast protein STI1." THE JOURNAL OF BIOLOGICAL CHEMISTRY 25 APR 1992, vol. 267, no. 12, 25 April 1992 (1992-04-25), pages 8485-8491, XP002510163 ISSN: 0021-9258 abstract	1-14